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THE VIRUSES

Volume 3

ANIMAL VIRUSES

THE VIRUSES

Biochemical, Biological, and Biophysical Properties

Edited by

F. M. BURNET
The Walter and Eliza Hall Institute of Medical Research Melbourne, Australia

W. M. STANLEY
Virus Laboratory University of California Berkeley, California

Volume 3

ANIMAL VIRUSES

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Contributors to Volume 3

F. M. Burnet, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

S. G. Anderson, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Alfred Gottschalk, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

F. B. Bang, Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland

Alick Isaacs, National Institute for Medical Research, London, England

R. Walter Schlesinger, Saint Louis University School of Medicine, Saint Louis, Missouri

Frank L. Horsfall, Jr., The Rockefeller Institute for Medical Research, New York, New York

Frank Fenner and John Cairns, Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

T. Francis, Jr., University of Michigan, Ann Arbor, Michigan

Howard B. Andervont, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Kenneth M. Smith, Agricultural Research Council, Virus Research Unit, Cambridge, England

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Preface

Experimental biology at the present time is approaching a particularly interesting stage. It is probably true to say that in recent years biochemical concepts have dominated all general thinking on the fundamental problems of biology. There has been an uninterrupted succession of important discoveries and there is as yet no sign of any drying up of the flow of discovery. It is orthodox to believe that the way is now open to a comprehensive understanding of the basic living process in terms of biochemistry, with perhaps only an inspired continuation of progress along present lines being necessary. There are some, however, who are more impressed with the mounting difficulties of applying the methodology of chemistry to the complex macromolecules of living systems and their interactions than with the inevitability of their being overcome.

Clearly the crux of biological thought today is the applicability of chemical and physical approaches to the typical macromolecules, protein and nucleic acid, of living substance. In this context virology seems to occupy a key position among the biological sciences. Viruses are the smallest biological units which manifest all the essential characteristics of life and many are now known to be built up only of nucleic acid and protein. With the development of new biochemical techniques useful in attacking the problems of macromolecular structure viruses have become the material *par excellence* for fundamental study. The very great discovery that nucleic acid preparations possessing virus activity can be obtained from virus-infected tissues and from pure viral nucleoproteins has focused attention on nucleic acid as the key material in virus activity, in genetic activity, and in the synthesis of proteins and of nucleic acids. It would appear, therefore, that nucleic acid structures contain the codes for the fabrication of every individual of every species. Since some viral nucleic acid preparations can be obtained quite pure, chemically as well as genetically, in lots of hundreds of milligrams, it is obvious that the viral nucleic acids offer an especially favorable and perhaps unique possibility of breaking the code and of approaching the synthesis of a replicating structure. These represent great challenges in virology and are, of course, of the greatest importance to science and to mankind.

There have been many systematic compilations of knowledge on viruses as agents of disease in man, in animals, or in plants. To our knowledge, however, the present work is the first to be published in English in which a systematic attempt is made to cover the significance of experimenta work on viruses for general problems within and on the borderlines of biochemistry, biology, and biophysics. Since it would be impossible for one or two persons

to write authoritatively about all of the important aspects of virology, it has been necessary to seek the assistance of experts in different areas of virology. The editors have been quite fortunate in securing contributions from 34 of the leading virologists in 8 different countries. Almost two-thirds of the contributors are from American laboratories and this mirrors with reasonable accuracy the relative activity in virology during the past several years. However, there is presently a great upsurge of research activity in virology in several countries and significant new information is now almost world-wide with respect to source.

The plan of "The Viruses" has deliberately followed that of the works on "The Proteins," "The Nucleic Acids," and "The Enzymes" published also by Academic Press. In this treatise on "The Viruses" we are concerned essentially with the chemical and physical characteristics of viruses and with the processes associated with their multiplication in the cell. In general we are not concerned with manifestations of viral infection in multicellular organisms or in populations of susceptible hosts except insofar as they provide information about processes at the cellular level. It is manifestly impossible to treat comprehensively of viruses without consideration of their behavior at the genetic level. Particularly with the bacterial viruses there is now much information on record on recombination between viruses and on interaction between the genomes of virus and host cell. In this region we may well find the material from which will come eventually an understanding of the relationships between the genetic and chemical approaches. The discovery of infectious nucleic acid preparations represents a major start in this direction.

It would not be realistic to separate the academic approach to virology sharply from the clinical. In the last analysis the prevention or cure of virus disease will depend on properties of virus and host cell. In the past, success in control has depended almost exclusively on the use of procedures at the immunological or epidemiological levels, but if these are to be refined and fully understood much use will have to be made of the information provided by the essentially theoretical studies which the present work has been designed to systematize and display. Consider, for example, the new immunological problems that one may encounter if, as may emerge, infectious nucleic acid moves directly from cell to cell. This treatise provides the information and the interpretation of this information that will be necessary for a rational experimental approach to such new problems.

One of the main difficulties the editors have encountered has been one inherent in all attempts at biological generalization, namely, the diversity of the material that is available for study and the widely varying intensity and success with which different sections of that material have been studied. It will be found, for instance, that a large proportion of each of the three

volumes is concerned with the properties of one plant virus (TMV), one bacterial virus (T2) and three animal viruses (vaccinia, influenza A, and poliomyelitis viruses). There are large numbers of other viruses in each of the main categories and undoubtedly many show or will show highly significant differences in behavior from those which for one reason or another have been chosen as prototypes. Perhaps one of the important functions of the work will be to show where such significant differences can most hopefully be looked for. Clearly there is no lack of scope for further work in virology.

Melbourne, Australia F. M. Burnet

Berkeley, California Wendell M. Stanley
December, 1958

Chapter I

Animal Viruses: A Comparative Survey

F. M. Burnet

Walter and Eliza Hall Institute, Melbourne

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Under the name of animal viruses, we are compelled to include what is manifestly a heterogeneous collection of agents. This makes it extremely difficult to provide a comprehensive general picture of the process of virus infection in mammalian and avian hosts. It seemed desirable, therefore, to begin the present volume with an introductory chapter which would allow some consideration of the main functional types of virus from a comparative viewpoint. Most of the points to be mentioned are based on factual material presented in later chapters, but investigation of some important groups of viruses has been so limited that very little use of the results can be made in attempts to analyze specific aspects of infection. In this chapter we are concerned with defining the range of infectious agents covered by the term animal viruses and with presenting a comparative survey of the main groups with some necessary attention to classification and possible evolution. In this way we can hope to supply a background against which the detailed accounts of structural and functional attributes of animal viruses which occupy the rest of this volume can be coordinated.

I. Definition of Animal Viruses

In attempting to define animal viruses, we immediately encounter characteristically biological difficulties. From our point of view the most satisfactory definition is probably the following:

An animal virus is a replicating agent or microorganism which is capable of growth and replication only within the living cells of some warm-blooded vertebrate, and which is composed of, or contains, protein and nucleic acid carrying patterns of specificity distinct from any produced under the genetic control of the host alone.

The first difficulty arises over the fact that this definition would include rickettsiae which, by convention, are excluded from the group, although the rather closely related agents of the psittacosis group are always known as viruses. One good reason for this is the undoubted resemblance in some aspects between the psittacosis viruses and the poxviruses. Both produce, for instance, a lipid-containing hemagglutinin. There are probably some virologists who would regard all three groups, rickettsiae, psittacosis group, and poxviruses, as small microorganisms of obligate intracellular parasitism but in all other respects differing from "true" viruses. However, it is a historical fact that most of the fundamental concepts of virology arose from the study of the variola-cowpox-vaccinia system; if a systematist were seeking a type species for the whole assemblage, he would be compelled to choose vaccinia virus. By one means or another, our definition must include the poxviruses.

In attempts to make a reasonably satisfactory compromise, a number of possible additions to the definition have been suggested:

1. The average value for the longest diameter of the infective particles does not exceed 300 mμ (millimicrons).
2. A virus is insusceptible to the action of antibiotics of the tetracycline, penicillin, and sulfonamide groups.
3. Viruses show no evidence of any intrinsic intermediary metabolism.
4. An eclipse phase can always be recognized in the course of virus infection.
5. Lwoff (see Chapter 5, Volume II) suggests that the term virus should be confined to viruses having only one kind of nucleic acid.

None of these is particularly satisfactory in practice. The quality suggested may be one requiring elaborate experimentation to establish, while other qualities may be unsuitable for application to certain strains. Some influenza strains, for instance, may produce infectious filamentous structures up to 10μ long, while sulfonamide- or penicillin-resistant strains of psittacosis group viruses can be obtained.

Despite Lwoff's contention that a virus is a virus and not to be thought of as a degenerated bacterium or anything else, it is very difficult to feel in any way confident that in the animal viruses we are dealing with a group of biologically related agents. In inviting contributors to write on this or that aspect of the animal viruses, the editors felt it necessary to ask that, in general, three viruses should be considered as prototypes—vaccinia, influenza A, and polioviruses. There were two obvious reasons for this: the first, that it was essential that at least three major types of virus should be considered

in the discussion of general aspects of structure and functional activity; and, the second, that for various reasons these were the three virus types which had been most extensively investigated in the laboratory.

This is clearly an arbitrary arrangement and where information available is relevant other types are freely discussed. An ideal presentation would probably have to provide at least three additional prototype viruses representing, respectively, the psittacosis group, the adenoviruses, and the arthropod-borne viruses (arborviruses), typified by yellow fever and western equine encephalitis viruses.

II. A Basis for the Classification of Animal Viruses

Within the last ten years a beginning has been made in the classification of the better-known animal viruses. Definitive accounts of the classification accepted at the Sixth International Congress of Microbiology, Rome, 1953, for several of the groups, have now appeared (Andrewes *et al.*, 1955; von Magnus *et al.*, 1955; Fenner and Burnet, 1957).

Since the Rome discussions, a number of new points that seem relevant have arisen and it may be of value to make some comments in regard to each of the groups which have been accepted as appropriate for naming, either at the Congress or by unofficial action since.

A. Psittacosis Group

This group was deliberately excluded from the discussion of virus classification at the Rome meeting. It has, however, some marked resemblance to the poxvirus group and has been regarded by the editors as falling within the scope of this work.

There is no uniformity of opinion as to the number of species contained in the group or whether the name *Miyagawanella* used in Bergey is a valid one (Meyer, 1953; Rake, 1953).

Psittacosis virus proper is typical of a widespread group of avian pathogens and there is an almost equally wide range of forms parasitic in mammalian tissues, of which the virus of lymphogranuloma venereum has been most extensively studied.

They all show a complex intracellular development, producing in early stages forms much larger than the elementary bodies, which represent the final infective product liberated from the cell. These elementary bodies, when prepared for electron microscopy, show a central "nucleus" surrounded by a loose-fitting, wrinkled skin. The central region is Feulgen-positive and both DNA and RNA have been shown to be present in purified virus particles (Zahler and Moulder, 1953).

Psittacosis group viruses approach the rickettsiae in being sensitive to the action of tetracycline antibiotics and in their staining reactions, but differ

in the failure so far to show any dehydrogenases of the type found with rickettsiae (Moulder and Weiss, 1951). They resemble the poxviruses in the type of hemagglutinin they produce.

B. Poxvirus

With vaccinia virus as the type species there can be no doubt that those viruses with infective particles of the same large size and characteristically complex morphology, and showing serological relationship to vaccinia, form a closely knit group. This includes variola vaccinia, mousepox (ectromelia), Jennerian cowpox, and rabbitpox viruses. A second group includes the avian poxviruses, of which classic fowlpox and canary pox have been the forms most studied. Sparrow pox was described by McGaughey and Burnet (1945) and there can be no doubt that there are many more strains of related viruses in wild birds. In Australia, French and Reeves (1954) isolated from mosquitoes several strains which appear to represent three types of fowlpox-like virus, one being standard virus and the other two presumably enzootic in wild birds. Australian magpies (*Gymnorhina*) have been found infected in the wild (Fenner, personal communication). As far as they have been studied, the avian pox viruses have no serological relationship to the vaccinia group and produce no hemagglutinin.

The third group comprises the rabbit myxoma and fibroma viruses, to which can probably be added Kilham's (1955) squirrel fibroma and the disease of deer described by Shope, and perhaps a condition recently observed in West African monkeys (Andrewes, personal communication). Some doubts were raised about the correctness of the inclusion of these viruses in the poxvirus group, but morphologically they are virtually indistinguishable from vaccinia (Fenner, 1953). The acutely fatal character of myxomatosis has its equivalents in malignant smallpox or canary pox, and transfer by mosquitoes is also the most important way in which the avian poxes are spread.

Molluscum contagiosum still awaits adequate study and is in the group simply by virtue of morphological resemblance. The same holds to varying extent for a variety of other mammalian poxviruses that have been only superficially studied.

Detailed study within the group has concentrated mainly on vaccinia virus, but it is probable that in all essentials the other types conform. The picture presented is very different from that obtained with the other well studied viruses. In two respects vaccinia virus resembles psittacosis virus. Both contain DNA and produce a nonviral hemagglutinin of similar character with lipid as an important component. RNA is present in viruses of the psittacosis group and has not been reported for vaccinia virus, but further study of this point is probably required. The other feature of interest for any

comparative approach to the animal viruses is the morphological and biochemical complexity of the virus particle. Probably very few virologists would dispute the legitimacy of describing vaccinia virus as a microorganism, although some might regard this admission as justifying the rejection of the poxvirus group as viruses at all.

C. Herpesvirus

It is unfortunate that the very interesting group containing the viruses of herpes simplex (*hominis*), B (*simiae*), and pseudorabies (*suis*) has not been closely studied in regard to the properties of the infective particles. It seems likely to be relatively close to the poxvirus group but there is insufficient evidence to justify discussion here. The same holds for another relatively large virus, that of rabies.

D. Myxovirus

The myxovirus group includes the two classical influenza types A and B, mumps virus, and a growing list of minor respiratory pathogens of man, as well as two classic avian diseases, fowlplague and Newcastle disease, and possibly some other avian types. There is more than a likelihood that extensive study of domestic and wild mammals would disclose other myxoviruses. It is a very interesting situation that complement-fixing antigen of influenza A is known to be present in the human virus, in swine influenza virus, in fowlplague virus, and in virus responsible for infection in Swedish horses (Heller *et al.*, 1956). It appears that this is a highly versatile stock. The diagnostic characters of the group include the rather variable size of spherical infective particles, around 100 mμ in diameter, and the common occurrence of filamentous forms of virus. All react with mucoproteins on the surface of cells and in solution under appropriate conditions and carry an enzyme capable of splitting neuraminic acid or related components from the reactive mucoproteins. The nucleic acid content is low and wholly RNA.

E. Adenoviruses

We can feel confident that within a year or two the adenoviruses will take their places as one of the key groups for fundamental investigation of viral function. Effective experimentation is, however, only in an early stage. The group was defined in terms of the characteristic cytopathogenic effect seen in tissue cultures of monkey kidney or HeLa cells, and the existence of a complement-fixing antigen common to the group (Enders *et al.*, 1956). Fourteen serological types have been described on a basis of specific neutralization by antiserum in tissue culture tests. The unique feature of the group is its production of what appear to be crystalline aggregates of virus within the nucleus of affected cells.

F. Arborviruses

No official nomenclature for this group of arthropod-borne viruses has yet been accepted. It is, however, already clear, from the work of Casals, Theiler, and others, that these viruses typified by those of yellow fever and western equine encephalomyelitis form a relatively homogeneous natural group (Report, 1956). Physically, the infective particles of all the well-studied members of the group are spherical and about 30 mμ in diameter. Those of western equine encephalomyelitis contain a high content of lipid, and all their nucleic acid is RNA. They all can be shown to act as hemagglutinin for erythrocytes from day-old chicks when the tests are made under strictly controlled physical conditions. By hemagglutination-inhibition tests most of the well-known viruses fall into one or another of two serological groups: A, containing the equine encephalitis viruses and a few others, while B contains a large number of important human pathogens, including yellow fever, dengue, and Japanese B viruses. Two other serological groups (C and D) have been demonstrated and there are still a number of viruses, too inadequately studied to be sure of their relation to the better-known forms. The encephalomyocarditis (EMC) viruses may represent a related group.

G. Enteroviruses

Recent findings have obscured the demarcation lines originally drawn between the polioviruses, the Coxsackie viruses, and the ECHO * viruses and, despite the great differences in their medical significance, it seems likely that all these denizens of the human intestinal tract fall into one natural group. Polioviruses can be adapted to infect suckling mice and to lose all capacity to produce lesions in the central nervous system of rhesus monkeys. Serologically similar viruses may include strains, some of which show typical Coxsackie lesions in suckling mice, others which can be propagated only in tissue culture and would, in the absence of that serological relationship, be classed as ECHO viruses. This interrelationship has recently been regarded as adequate to justify referring to the whole group as enteroviruses and we shall adopt this name (Committee Enteroviruses, 1957).

All of these viruses have small, spherical, infective particles which are uniform enough to pack into crystal lattices. They are highly resistant to environmental damage and probably contain only protein and RNA as significant components.

To date, attention has been mainly concentrated on human material, but it is already clear that similar forms can be isolated from bovines, monkeys, and other mammals. The fact that foot-and-mouth disease viruses are of the

* Enteric cytopathogenic human orphan.

same particle size and are pathogenic for suckling mice would almost demand that these should be included in the group.

H. Other Groups

Apart from these major groups of virus, there remain many other forms which have not yet been found susceptible to classification. A few may be mentioned as perhaps representative of important groups.

1. The avian viruses, producing intranuclear inclusions, of which infectious laryngotracheitis is the prototype.

2. Measles and canine distemper viruses, which may perhaps be the two conspicuous members of a much wider group.

3. Vesicular stomatitis of cattle and swine with the two serological types, Indiana and New Jersey.

4. It has been suggested that there may be a common character to the viruses of infectious and serum hepatitis, rubella, and infectious mononucleosis.

5. The virus of neurolymphomatosis of fowls is accepted by Beard and others as the prototype of a wide range of viruses responsible for proliferative lesions in domestic poultry. It might be one of the most illuminating discoveries in virology to find what form the ancestral forms of these viruses take in wild birds.

This still leaves the agents of one important human disease, rabies, and many diseases of domestic and wild animals unclassified. Research has naturally been concentrated on viruses responsible for human disease and on those producing economically serious disease in domestic stock. Among these the availability of convenient laboratory hosts has been largely responsible for the choice of a very small proportion for detailed analytic studies.

III. The Comparative Picture

The picture that seems to emerge of the animal viruses as a whole is, broadly speaking, not very dissimilar to that of any wide assemblage of higher living forms, mammals or molluscs, for example. There are clearly demarcated groups with basic similarities which have obviously flourished and evolved to fill the available ecological niches. With the development of tissue culture methods, we have been freed of the necessity of demanding pathogenicity before a virus could be recognized and there has been an almost embarrassing flood of virtually nonpathogenic forms, closely related in many instances to viruses of classical pathogenicity. The extraordinarily large number of "wild monkey kidney viruses" that Hull and others (1956) have described and the various forms that have been isolated from experimental mice in the last twenty-five years make it obvious that there are vast

numbers of virus types yet to be examined. Until a much larger fraction of the whole universe of viruses living in mammalian cells has been made available for study, any general survey will be heavily biased toward a consideration of types actively pathogenic for human beings and their less dangerous congeners.

Even here, however, some order can be seen. There is at least a suggestion that ecological niche and physical structure have a significant relation to one another, with the implication of common descent and evolutionary radiation in the history of the group.

The poxviruses are primarily skin pathogens, multiplying in epidermal cells and spread by mechanical transfer to traumatized skin, often by mosquito or other biting vector. Depending on strain and host species, an additional viremic phase may or may not be manifest. Their complex structure, presence of intrinsic components like copper and biotin, which point to an autonomous intermediate metabolism of some sort, large size, and possession of DNA point almost categorically to evolution by parasitic degeneration from a bacterial form.

The psittacosis viruses have even closer resemblance to small bacteria and it would be easy to make a case for choosing *Brucella suis*, *Coxiella burnetii*, psittacosis virus, and vaccinia virus as modern representatives of the general line of evolution to vaccinia virus.

The myxoviruses give every indication of being preeminently parasites of respiratory mucous membranes with, however, potentialities for invasion and generalized spread, as in fowlplague and mumps. They differ from all the other groups in the relatively high content of host cellular material that is included in the infective particle. This statement would perhaps be questioned by some, while others might remark that it may only be because technical approaches to the influenza virus studies have not been applied to other viruses that the apparent contrast exists. Everything, however, points strongly to an active participation of the surface layers of the host cell in the fabrication of the surface of the virus particle, whether spherical or filamentary. Another striking feature is the lack of uniformity of size and the ease with which functionally and/or morphologically anomalous forms can be produced. The infective particles contain only RNA and are unique among viruses in carrying an active specific enzyme, neuraminidase, presumably as a surface component of the particle.

The arborviruses have exploited the same niche for survival as an immense group of parasitic protozoa, most of which are also obligate intracellular parasites. It is of interest that the infective sporozoites of malaria injected by the mosquito are very small, although of course much larger than any virus, that they are stored in the insect's salivary glands, and that on entry into the body they first undergo extraerythrocytic development in liver cells.

The resemblance to the life cycle of yellow fever may be only superficial, but it at least underlines the ecological similarity of the two sorts of agents. Most of the arbor group are mosquito-borne viruses of characteristically tropical habitat with an extraordinary catholicity of host cells in which multiplication can occur. Apparently almost any mammal or bird can be infected, usually without symptoms, and a wide range of Culicine mosquitoes will allow multiplication in the alternate phase.

There is still scope for further work on the physical nature of the infective virus particles of this group. Though one can recognize the great technical difficulties involved, it would be of the greatest interest to know whether the infective form in the mosquito salivary gland has the same physical form as that produced in mouse brain or chick brain or chick embryo tissues. The only features especially characteristic are the presence of RNA only, the high content of lipid, presumably on the surface, and the presence of not very robust hemagglutinating power.

The small intestinal viruses, as observed in man, have as common ecological features a concentration of spread in the warm season of the year, transfer by ingestion of fecally contaminated material, and the development of substantial but rather highly specific immunity following infection. These three features are responsible for almost all the well-known aspects of poliomyelitis as a disease. Broadly speaking, the capacity of some of these strains and not others to invade central nervous system, muscle, or other organs in a proportion of individual hosts is a biological accident of only medical significance. The existence of herpangina in man provides a link to justify the extension of the ecological niche to the whole gastrointestinal tract and so allow the inclusion of foot-and-mouth disease viruses in the enterovirus group.

These are the simplest of all viruses—uniform spheres which pack smoothly into crystal lattices containing no other significant components than protein and RNA (Schwerdt, 1957). Their immunological behavior suggests that the surface of the particle is of protein. All that have been examined seem also capable of producing in infected cells material of smaller particle size than the virus, with immunological specificity related to virus and not to the host. There are hints from genetic work (Sprunt *et al.*, 1955) that phenotypic mixtures containing two distinct antigens can be produced in mixed cultures.

It is probably too early to make more than tentative suggestions about either the ecological or physical characters of the adenoviruses. In man they are parasites of mucous membranes which seem to have a special predilection to produce proliferation of lymphoid cells in submucosal situations and, at least under some conditions, to persist for long periods in this situation. There is as yet no adequate ground to discuss the relation of the intracellular crystalline structures to the infective particles.

IV. Evolution of the Animal Viruses

Any thoughts about the evolution of the animal viruses must depend on our understanding of their present ecological and functional attributes, and they must be subject to modification with every increase in knowledge. Their only importance is to provide an occasional hint that may point toward an area that has been inadequately studied.

For many years the Green-Laidlaw theory of parasitic degeneration has been the only hypothesis of viral evolution that has been seriously discussed. This does not necessarily mean that it is correct. Broadly, there are three possible hypotheses: (1) that the specific patterns characteristic of viral protein and nucleic acid are determined by a genetic mechanism which can be traced back to that of some autonomous group of microorganisms; or (2) that the genetic mechanism was at some stage in its descent part of the genome of an organism related to the present host or possibly to an insect vector. The third possibility is that viruses descend from an extremely primitive precellular stock of protoorganisms which could only survive as intracellular parasites once modern organisms had appeared and which have been viruses since the pre-Cambrian era, changing host and habit as the course of evolution dictated.

On any of these concepts, once a virus species has found it possible to survive by transfer from one host individual to another, the possibilities of change by mutation and selective survival are introduced. Having regard to the genetic lability of such viruses as have been studied in the laboratory and the rapidity of their generation time, one would expect (a) that, on transfer to a new environment, adaptation to the form optimal for survival would take place rapidly, and (b) that once a satisfactory host-parasite relationship had been evolved the situation will remain virtually unchanged indefinitely.

The only real virtue of the Green-Laidlaw theory is that it allows the logical inclusion of psittacosis and poxgroup viruses among the animal viruses. Neither of the other two hypotheses makes sense for these. The small RNA viruses which have no other components than nucleic acid and protein can be interpreted either as the limiting stage of parasitic degeneration or as equivalent to, and perhaps developed from, the smallest protein synthetic units found in all types of cells. The nature of the microorganisms from which viruses are presumed to have descended can only be guessed at. It has already been suggested that the poxviruses and psittacosis group viruses may be descended from bacteria not unlike brucella, with rickettsiae as an intermediate step. Andrewes (1957) has suggested that the rickettsiae are primarily parasites of insects and that much of the evolution of animal viruses may have taken place in the terrestrial arthropods. The arborviruses could also be regarded primarily as having evolved in insects, but more probably from protozoal than from bacterial ancestors. This concept may be found

helpful when technical methods become available for a comparative functional study in insects of the viruses which are potential insect-pathogens (see Chapter XV) and the arborviruses. This is perhaps the most promising untilled field still available to the virologist.

The myxoviruses seem to be characteristic parasites of mucous membranes and their possession of a highly active neuraminidase is suggestive of a distant bacterial origin. The absence of such enzymes in vertebrate tissues makes it unlikely that it could have been evolved in any other fashion.

V. Is There Any Functional Unity among the Animal Viruses?

This survey has, in a sense, already answered this question by demonstrating that there are at least six successful patterns of animal virus structure; the body of the work will make it clear that for none of the three prototypes chosen from these have we more than a crude intellectual model of the process of virus reproduction within the cell. There is, however, one extremely important feature common to all the viruses—that they are parasites of mammalian or avian cells. Further, two types of cell, the chorionic epithelium of the chick embryo and the HeLa line of human cells in tissue culture, will, between them, allow examples of all groups to multiply freely. All the viruses seem, therefore, to have been adapted by evolution to make use of the standard structural and metabolic outfit of the vertebrate cell for their effective reproduction. Differences in the susceptibility of different cells are probably to be related mainly to specialized differentiations appearing in the course of development. It is highly significant that embryonic cells and dedifferentiated (malignant or pure line tissue culture) cells are more widely susceptible to allow free multiplication of, and cytological damage by, viruses.

This is the main justification for a preliminary and very provisional attempt to provide an orientating discussion of the relationships of the main virus types to the mechanism of the standard, undifferentiated, vertebrate cell. As a starting point it is probably best to adopt Hotchkiss's (1957) suggestion that, although the invading virus necessarily makes use of the whole metabolic machinery of the cell, it actively interferes with and deviates the metabolic sequence only at one restricted region of the sequence. The region of interference may differ from one virus to another and, in all probability, is closely correlated with the differences between the major groups of viruses. In a search for the nature of the interference that leads a cell to synthesize virus material instead of its own structure, we are virtually obliged to use as a guiding thread the nature of the viral nucleic acids. The content of the virus particles in nucleic acid is tabulated in Table I for the six groups we have

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TABLE I

NUCLEIC ACID CONTENT OF SIX VIRAL GROUPS

DNA and RNA	DNA	RNA
Psittacosis group	Poxvirus (?) group Adenoviruses	Myxovirus group Arborviruses Enteroviruses

been concerned with. The possibility that poxvirus should be in the first (DNA and RNA) group is not wholly excluded.

The most significant chemical finding relevant to the point at which interference by a virus can occur is derived from the study of the action of the ribosyl-benzimidazole derivative, DRB. This inhibits the early stage of influenza virus production and shows an inhibitory influence on the uptake of labeled amino acids by thymus nuclei with almost the same time sequence. In the latter case, Mirsky and Allfrey (1957) consider that the effect is to block the synthesis of RNA, which is needed before protein synthesis is possible. Once RNA of appropriate specific pattern has been synthesized, it appears that protein synthesis can go on without the necessity for concomitant further synthesis of RNA.

One way of looking at the RNA-containing viruses could well be to concentrate on the ribonucleoprotein common to them all. There is very much to suggest that, as Polson and others have claimed, soluble complement-fixing antigens from viruses tend to be about the same size, 10–15 mμ, as is postulated for the primary protein synthetic mechanism by electron microscopists. Pallade (1957) for instance, considers that spherical units of RNA and protein about 150 Å in diameter, represent a standard form of protein synthetic mechanism in animal, plant, and bacterial cells. A poliovirus particle could be pictured, according to Crick and Watson (1956), as a firm, symmetrical structure of 12 such units (or 60 smaller ones), containing only ribonucleic acid and protein. Western equine encephalitis virus may represent a similar complex which, in the process of formation, takes up a large proportion of lipid, and influenza virus as a loose accumulation of about the same number of primary units contained in an enclosing membrane of complex character which incorporates both viral protein and host cell constituents.

On this view, the essential interference by the virus might well be the intrusion of virus RNA into the nucleus where virus RNA-protein complexes, which in the appropriate metabolic environments can produce either RNA or protein, are laid down. The possibility that the new synthetic units are in some fashion contrived from cell units already with a defined function might allow for the synthesis of viral complexes or components with the same sort

of potentiality for special association with lipids or mucoproteins as some cell proteins possess. Along such lines one could think of the sequence of increasing incorporation of host cell components seen in the series poliovirus, arborvirus, and myxovirus.

Such a picture will undoubtedly be discarded as knowledge of the synthetic mechanism of cells increases. But we can be certain that, with each development in the field of macromolecular chemistry, it will become clearer where the intrusion of the virus into the cell affects the metabolic processes. It is equally evident that the behavior of viruses may provide one of the most important tools for the continuing exploration of the broader field of protein and nucleic acid in general. These two statements are virtual truisms, but it is less widely recognized that the behavior of viruses may be equally relevant to the understanding of the differentiation of parts and morphological control within the cell. There are many hints, in addition to those mentioned above, that there are special relationships between the host cell surface and virus infection. One way of looking at both the proliferative stimulation provided by some types of virus infection—the Shope fibroma, for example—and the oncolytic effect of other viruses is to postulate specific effects at the cell surface (Burnet, 1957). In this general field, the differences between the various groups of animal viruses may be more illuminating than their resemblances.

Although only this sketchy outline is possible for the RNA-containing viruses, the position with the DNA viruses is even worse. It may well develop that analogies to bacteriophage behavior will be helpful in the interpretation of viruses like the adenoviruses and Shope papilloma virus. There is, however, the very important gap in phage studies that we do not know with certainty whether or not phage protein pattern is determined directly by DNA or through some type of intermediary mechanism involving protein, RNA, or both. From the point of view of fruitful analogy with animal viruses, there is the further disadvantage that T-even phages, which have alone been really intensively studied, possess DNA uniquely different in its content of 5-hydroxymethyl-cytosine and glucose from any other DNA in the whole range of life.

The approach to an understanding of the relationship of the larger viruses of the psittacosis and poxvirus groups to the infected cell will probably draw more from bacterial physiology than from the behavior of the smaller RNA viruses. Both types have a clearly demonstrable limiting membrane and a central body of nuclear character containing DNA. In the closely studied poxviruses, Peters (1957) comes to the conclusion that, despite the disparity in size, the general structure of the vaccinia virus nucleus and its reactions to enzymatic treatment is closely reminiscent of bacterial nuclei, such as those in spores of *Bacillus megatherium*. If this is the case, these viruses will have

to be accepted as of a higher order of organization and much less attractive material for fundamental studies on virus action than the smaller RNA-containing forms.

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Chapter II

The Initiation of Infection by Animal Viruses

F. M. Burnet

Walter and Eliza Hall Institute, Melbourne

The first step in the process by which virus and susceptible cell interact to produce a new generation of virus must necessarily be concerned with the entry of the infective particle into the substance of the cell. The corresponding sequence of events in infection of the host by a bacteriophage has been intensively investigated, but in the field of animal viruses very little is accurately known and direct experimentation has been almost wholly limited to the myxovirus group. A wide range of viruses appears to be dependent on some degree of traumatization to allow initiation of infection. It is characteristic of mammalian and avian cells other than those exposed to the external environment and its extensions that a large proportion will take in particulate material nonspecifically and, provided the cell in question is susceptible, no problem of a mechanism of entry arises.

On general grounds, it is only in viruses which, to survive in nature must infect intact surfaces exposed to the environment, that we might expect to find specific mechanisms for the initiation of infection. Of such viruses pathogenic for man, the influenza viruses and the enteroviruses, including polioviruses, have each some claim to be considered in this light.

An immediate and characteristic difficulty of experimental virology is encountered in the dilemma that the significant characters of a virus are those which have evolved to allow its survival in nature, but that the only characters which can be accurately studied are those relevant to infection of some uniform but unnatural host—the allantoic cavity or cells in monolayer tissue culture. Findings from the experimental systems will undoubtedly have general relevance to the problem, but they will need careful scrutiny before being used to interpret clinical or epidemiological phenomena.

Discussion of the initiation of infection of the animal cell has been dominated by the analogies drawn from hemagglutination reactions with influenza and other viruses of the group. Chapters III(I)B and IV in this volume deal with biological and chemical aspects of hemagglutination, but they touch only very indirectly on the mechanism of entry into the susceptible cell. In a very real sense they represent accounts of what may be only two minor aspects of the process that have come into prominence only because of the availability of suitable experimental approaches.

If the reality of the eclipse phase is accepted, the phase of initial infection of the cell could be defined as extending from first contact from the environment to the stage at which loss of morphological form and infectivity marks the beginning of the eclipse phase. Experimental approaches to the study of this phase are not as yet particularly effective, even when highly susceptible and uniform host cells are used. They include:

1. Direct examination of cell sections by electron microscopy.
2. The rate and completeness with which virus is adsorbed by a known area of susceptible cell surface.
3. The susceptibility of attached virus to liberation by enzymes or inactivation by immune serum at various time intervals.
4. The effect of soluble substances, e.g. mucoprotein inhibitors, in preventing adsorption.
5. Modification of the susceptible cell surface by agents such as the receptor-destroying enzyme (RDE) or metaperiodate ion.

Some account will be given of the application of these approaches to (a) influenza, fowl plague, and Newcastle disease viruses (NDV), and (b) polioviruses and other viruses of the intestinal group.

Myxovirus. This is the only group of viruses for which some direct evidence has been obtained by electron microscopy. Adams and Prince (1957) have recently published pictures of NDV particles adsorbed to the surface of "semisusceptible" cells of Ehrlich ascites tumor in mice. The virus particles appear intact, except for the disappearance of the outermost coat of the particle, perhaps as a result of having fused with or been "wetted" by the cell substance.

In the standard experimental situation of the allantoic cavity, it is usually found that only 50–90 % of the inoculated virus is adsorbed before new formation and liberation of virus commences. No fully satisfactory explanation of this incompleteness of adsorption has been provided, but the presence of potentially inhibitory mucoproteins in the allantoic fluid may well be the most important factor.

When attachment occurs, virus is temporarily removable by the action of RDE and neutralizable by immune serum. According to Ishida and Ackermann (1956), the first step with PR8 and chorioallantoic membrane pieces is to develop stability to RDE while still susceptible to inactivation by antibody. At 37°C., a second step rapidly follows by which immune serum becomes ineffective. In rather similar experiments with Ehrlich ascites tumor cells and the strain neuro-WS, Wagner (1955) found good adsorption of virus at room or refrigerator temperature and easy liberation by RDE. With active virus about 2 hours is required for union to become irreversible. When heat-killed cells are used, virus remains indefinitely elutable by RDE, indicating an active function of the cell in producing irreversibility. The possibility that

this irreversible union represents engulfment (phagocytosis or viropexis) (Fazekas de St. Groth 1948a) is strengthened by Ginsberg and Blackman's (1956) interesting experiments with guinea pig leucocytes. Treatment of polymorphonuclear cells with RDE destroyed their capacity to adsorb virus, but when normal cells had adsorbed virus, less than 10 % was liberated by subsequent RDE treatment.

It is usual to consider that the adsorption of virus to susceptible cell is by the same process as is concerned in virus adsorption to red cells. This is supported by the *in vitro* studies of Hirst (1943) with ferret lungs and of Fazekas (1948b) with mouse lungs. The latter showed, in addition, that pretreatment of the lungs with RDE destroyed their power to adsorb virus. Direct evidence that removal of cell surface receptors by RDE would protect mice against challenge by active virus was obtained by Stone (1948b). Similar results were reported for tests of protective power of RDE against allantoic cavity infection (Stone, 1948a). The effectiveness of pretreatment with RDE was, as might be expected, inversely related to the degree of adaptation and virulence of the virus for the particular test animal being used. There was only slight protection against highly virulent strains. Cairns (1951) found that RDE also showed a significant protective action against the neurotropic influenza strain NWS when inoculated intracerebrally.

It is clear that the presence of mucoprotein receptors susceptible to the neuraminidase of virus or RDE is propitious for infection, but even Stone's experiments do not indicate that they are necessary for infection. At one time it seemed likely that active enzymatic action was needed for infection to occur, but this is by no means established. Fazekas (Fazekas and Graham, 1949) have shown that infection can occur in embryos whose allantoic cells have been treated with periodate to render receptors insusceptible to the enzyme action. They consider that this excludes any active function of the enzyme in the process of infection—the entry of the virus into the cell being, in their view, due to nonspecific forces. Rubin (1957) on the other hand, using NDV and monkey kidney cells in tissue culture, is impressed with the parallel action of antiserum on enzymatic activity and infectivity, and calculates that the number of enzymatic sites on the particle is of the same order as the number of "infective sites." He considers that "these results support a role for the viral enzyme in the early steps of infection" and are not inconsistent with the view of Hoyle and Finter (1957) that some or all of the protein of the infecting virus particle remains on the surface of the cell. However, Wecker and Schafer (1957) find that when fowl plague virus labeled with P^{32} is used to infect tissue culture cells, both bound antigen with its contained ribonucleic acid and phospholipids are taken up by the cells. This strongly suggests that the whole virus particle is taken into the cell substance. Probably most workers would accept the weight of evidence as favoring a mechanism

of the viropexis type rather than a limited injection of the contents of the virus particle, as occurs with bacterial viruses.

As a reasonable current interpretation, it may be suggested that temporary attachment of the virus particle to cell surface takes place under the combined influence of electrostatic forces and specific union of viral enzyme with a sequence of prosthetic groups of surface mucoprotein. This provides an opportunity for viropexis to occur and trap the particle before enzymatic action has continued long enough to release it from the primary attachment. Whether the enzyme has any function after entry of the particle into the cytoplasm is unknown.

It is certain that the process of initiating infection is a complex one, even in the almost ideal circumstances of the allantoic cavity. Cairns (1957) studied the detail of infection in a group of embryos infected with a single infective dose of influenza virus. He found a very wide scatter in the lengths of time elapsing before liberation of new virus indicated that infection had been successfully initiated. The longest delay observed was 28 hours. Obviously, a wide variety of essentially accidental factors can increase the time needed before the conditions for effective interaction are found.

Enteroviruses. Very little information is available, except for work incidental to the establishment of satisfactory titration methods using the Dulbecco plaque technique. With standard monkey kidney cell monolayers, the process of adsorption is relatively slow. Dulbecco *et al.* (1956) show protocols indicating that exposure of cells to virus for 30 minutes at 37°C. gives about two-thirds the number of plaques obtained after exposure for 90 minutes, and tests on the supernatant fluids after adsorption give concordant results. Younger (1956) has published similar findings and showed that the unabsorbed fraction on subculture produced virus unchanged in its adsorptive qualities.

In view of the interest in living virus vaccines against poliomyelitis, it is most unfortunate that no appropriate experimental methods are available to measure adsorption to significant types of human cell, intestinal or neuronal. It is far from clear whether differences in virulence are related to differences in adsorption to cell surface or to factors associated with multiplication within the cell. No real advance in the understanding of virulence is likely to be possible, however, until an experimental attack on each aspect is possible.

The work on hemagglutination by the mouse poliovirus GD VII and inhibition of infection and hemagglutination by a polysaccharide from the mouse intestine (Mandel and Racker, 1953; Mandel, 1957) is referred to in Chapters III and IV. The indication from this, that there may be a specific relationship between some configuration of the virus surface and a mucopolysaccharide of the cell surface, seems to call for a serious study of the polioviruses generally from this point of view.

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Chapter III

Hemagglutination by Animal Viruses

S. G. Anderson

Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

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Hemagglutinating viruses can be conveniently described in three groups:

1. The myxovirus group, in which the virus particle itself is the hemagglutinating agent and also carries an enzyme (neuraminidase) which allows elution of virus with destruction of red cell receptors.

2. Viruses in which the virus particle is believed to be the hemagglutinin but which possess no eluting enzyme and produce no destruction of cell receptors on elution. This group includes most of the arthropod-borne viruses, the encephalomyocarditis (EMC) viruses, one member (GD VII) of the poliovirus group, and pneumonia virus of mice.

3. Viruses which, during growth in suitable cells, produce a hemagglutinin which is separate from the virus particle. This includes some of the poxvirus group and some strains of psittacosis and related viruses.

I. The Myxovirus Group

A. Historical

Hirst (1941) and McClelland and Hare (1941) independently reported that chick red cells were agglutinated by fluids from chick embryos infected with various strains of influenza virus. Hirst (1942a,b) explored the new field extensively, providing a sound basis for all subsequent work on the phenomenon. He showed: (1) that the hemagglutinin was identical with the infective particle, but (2) that, under the action of such agents as heat and formalin, infectivity could be destroyed with retention of hemagglutinating power, (3) that the spontaneous elution of hemagglutinin with stabilization of the red cell suspension closely resembled an enzymatic reaction, and (4) that hemagglutination could be inhibited by specific antibody and, to a less and variable extent, by some normal sera.

It was subsequently shown by Burnet (1942) that Newcastle disease virus (NDV) agglutinated red cells in similar fashion. Fowl plague virus was shown to be active by Lush (1943) and mumps virus by Levens and Enders (1945). The common property of these viruses to agglutinate red cells by adsorbing to mucinlike material on the cell surface was used to define the group myxovirus and to provide its name (Andrewes *et al.*, 1955).

B. Hemagglutination

1. *Relation to the Virus Particles*

Hemagglutination by standard infective preparations of influenza virus is due to adsorption of virus particles on the red cell surface; most workers picture the process as virtually a simple bridging between cells by the virus particles, with a resultant building-up of aggregates of cells. In addition to the virus particle hemagglutinin, a hemagglutinin associated with a still

smaller particle is produced in the course of infection with NDV (Burnet *et al.*, 1945; Granoff *et al.*, 1950; Granoff and Henle, 1954). The latter authors found that the small component was in higher concentration in the cells of the chorioallantoic membrane than in the allantoic fluid. It eluted from red cells more rapidly than standard virus and at lower temperatures. It had none of the special "binding" capacities of normal Newcastle disease virus, which are described later (Section I, C, 2, *d*). They considered that it might represent a precursor of the virus. What may be a similar small particle hemagglutinin can be obtained by disruption of at least some of the myxovirus group by prolonged ether treatment (Hoyle, 1952; Schafer and Zillig, 1954). In the case of fowl plague, the soluble hemagglutinin is a particle 10–15 mμ in diameter, composed of protein and carbohydrate but without nucleic acid (Schafer and Zillig, 1954). As far as they have been studied, the properties of the soluble hemagglutinin are similar to those of the virus particle hemagglutinin from which it was derived; further discussion in this chapter will be limited to the latter.

The identity of infective virus particle with hemagglutinin was established by demonstrating the correlation of infectivity and hemagglutinin titers under various conditions—in fresh preparations of complete virus (Hirst, 1942b; Liu and Henle, 1951), in the various stages of adsorption and elution with red cells (Hirst, 1942b; Taylor *et al.*, 1943), and on high-speed centrifugation (Lauffer and Miller, 1944; Sharp *et al.*, 1944; Taylor *et al.*, 1943).

With fully active virus, the amount of infective virus giving partial agglutination in a pattern test can be determined accurately. Fazekas de St. Groth and Cairns (1952) carefully defined titration methods for both hemagglutinin and infectivity, and gave a figure of about 10^6 ID_{50} for one hemagglutinating unit.

There is still controversy as to the number of particles, visible by electron microscopy, in the units of infectivity and hemagglutinin. Donald and Isaacs (1954) find that, in standard egg fluids with about 10^{10} ID_{50} per milliliter, there are approximately 10 particles per ID_{50}. Horsfall (1954), on the other hand, holds that with virus protected from the possibility of thermal degradation the infective unit corresponds to one visible, hemagglutinating particle. Levine *et al.* (1953) have claimed that a single particle is capable of holding two red cells in a dimer sufficiently stable to show a sedimentation boundary in the presence of unaggregated cells. This should provide an absolute method of measuring the number of virus particles in a given preparation. Tyrrell and Valentine (1957), on the other hand, find that one dimer occurs in the presence of ten particles of type A influenza, and Magill (1951) believes that there is always a proportion of free virus particles in the system, irrespective of elution through receptor destruction.

The size of the hemagglutinating particle has been determined by centrifugation and filtration studies, giving a particle diameter in the case of influenza of 80 to 100 mμ (Lauffer and Miller, 1944; Friedewald and Pickels, 1944; Elford *et al.*, 1936; Sharp *et al.*, 1944; Knight, 1946).

2. *Influence of Environmental Conditions*

a. Temperature. This is not critical, active agglutination being evident from 4 to 37°C. According to Miller and Stanley (1944), the hemagglutinin titer increases with increasing temperature, but, in general, the most important effect of higher temperature is to accelerate the process of elution. This may result, particularly with Newcastle disease virus, in the appearance of a prozone, if pattern-testing is being used. Sedimentation of the cells is more rapid at higher temperatures and this may be desirable with slowly sedimenting mammalian cells.

b. Ionic Environment. In 5 % glucose solution, PR8 does not adsorb to red cells. Lowell and Buckingham (1948) studied this system, adding various concentrations of sodium chloride to 5 % glucose solution. Adsorption of PR8 virus to red cells did not occur at concentrations below 0.003 *M* and hemagglutination failed below 0.043 *M*. The highest concentration tested, 0.583 *M*, allowed full agglutination. Strain LEE behaved similarly (Davenport and Horsfall, 1948) and Newcastle disease virus was shown to require an adequate ionic concentration by Sagik and Levine (1957). Burnet and Edney (1952) found that, for salts with monovalent cations, concentrations around 0.01 *M* were needed to allow hemagglutination by active virus. Divalent cations were more active, the end point being 0.004 *M* to 0.007 *M*. The nature of the anion used was immaterial, apart from a minor effect seen with calcium deionizing agents (Edney, 1949).

In influenza virus the optimal range of pH for hemagglutination is 6 to 8 (Miller and Stanley, 1944). With Newcastle disease virus, Sagik and Levine (1957) found optimal hemagglutination at pH 5.8 to 6.2, but good agglutination occurred over the whole range from pH 5 to 9, and also from 2 to 3. There were adsorption minima at pH 4 and 10.5.

3. *Influence of Species of Red Cell*

The red cells most widely susceptible to agglutination by myxoviruses are those from chicken, man, and guinea pig. Many other species provide cells agglutinable by some strains of virus but the type of agglutination varies. Comparative studies have been made by Clark and Nagler (1943) and Chu (1948c).

Newcastle disease virus has an unusual range of susceptible cells, Winslow *et al.* (1950) finding that the most susceptible species were cow, horse, and sheep. There were differences between different strains of NDV.

Even with one donor species, there may be differences in cell susceptibility to hemagglutination. With fowl cells there was only slight variation with influenza viruses (Miller and Stanley, 1944) but with mumps virus a fourfold range was observed (Beveridge and Lind, 1946).

Human type A influenza strains freshly isolated in the chick amniotic cavity frequently agglutinate human, pigeon, and guinea pig red cells to a much higher titer than fowl cells (Burnet *et al.*, 1942, 1945). Such strains are said to be in the O phase. They grow poorly, or not at all, in the allantoic caviry and have a lower avidity for the inhibitors in ovomucin and chick embtyo lungs (Stone, 1951).

Virus may often be retained in the O phase by passage at limit dilution but Hirst (1947) has reported difficulty with this. On repeated egg passage of O phase virus at low dilution, genetic mutants appear and rapidly dominate the population. This derived, or D phase virus, becomes the stable form on chick embryo passage. Intermediate forms between O and D—so-called δ and ω forms—may also appear during adaptation (Burnet and Stone, 1945a,b). D phase virus grows well in both the amniotic and allantoic cavities and agglutinates red cells of fowl, guinea pig, and human to virtually the same titer (Beveridge *et al.*, 1944).

Some strains, fully adapted to growth in the allantoic cavity, nevertheless fail to agglutinate fowl cells to a significant titer; an example is Ian O (Anderson and Burnet, 1947). This strain did produce a mutant, Ian D, which agglutinated fowl cells but did not overgrow Ian O in the allantoic cavity. In another instance, a highly egg-adapted virus, "Melbourne egg," developed a hemagglutinin pattern reminiscent of O virus in that it agglutinated fowl cells to lower titer than guinea pig cells (Burnet and Bull, 1944). A change superficially resembling the O-D transformation, but not based on genetic change, can be produced in WS (type A influenza) by alteration of ionic environment (Magill and Sugg, 1948; Burnet *et al.*, 1949).

A hint of a genetic change similar to the O-D transformation was found in type B virus by Burnet and associates (1944). A newly isolated strain developed a higher ratio between fowl and human cell agglutinating titers on repeated passage.

4. *Effect on Hemagglutination of Physical and Chemical Agents*

The biological activities of an influenza virus particle may be altered by a variety of physical or chemical agents. Susceptibility varies considerably from strain to strain and these differences have been valuable as markers in genetic experiments. In general, certain properties of the virus are altered or destroyed in the same sequence by each damaging agent, a sequence first established by the Henles in their work on ultraviolet irradiation.

a. Ultraviolet Irradiation. Ultraviolet light first destroyed the infectivity of PR8 strain of influenza in dialyzed allantoic fluid. Then the following properties were destroyed, in order—toxicity, ability to interfere, ability to immunize, ability to elute, hemagglutinin and complement-fixing antigens, probably 600 S before 30 S (Henle and Henle, 1947).

b. Aging. Hemagglutinin in allantoic fluid retained its full titer at 4°C. for several months (Miller and Stanley, 1944). Because of the frequent deposition of urates from untreated allantoic fluid, it is more convenient to dilute the fluid with saline before storage.

c. Heating. Myxovirus strains show wide variation in the temperature at which hemagglutinin is destroyed. Burnet (1951a), for instance, has reported two substrains of WS (influenza A 1933) which have thermal inactivation points of 67 (WSM) and 52°C. (NWS), respectively, when exposed for 30 minutes. Hanson *et al.* (1949) observed a similar wide range in susceptibility to thermal inactivation in a collection of Newcastle disease virus strains; some were inactivated at 56°C. in 5 minutes, others required up to 6 hours.

The eluting power of a virus is frequently destroyed by a degree of heating which leaves the hemagglutinin intact. This gives rise to so-called "indicator" virus (Stone, 1949b). For some strains, conversion to the indicator state occurs only if heating is carried out under special conditions, e.g., at pH 8.5 in the presence of citrate in the case of MEL virus.

d. Hydrogen Ion Concentration. At 23 and 4°C., the hemagglutinin of PR8 was best preserved at pH 7 in phosphate buffer, swine virus at pH 7 to 8, and LEE at pH greater than 9 (Miller, 1944). PR8 hemagglutinin was stable for 30 minutes throughout the range of pH 2.4 to 10.3 (Hirst, 1948a). Other factors concerned in the stability of hemagglutinin at different hydrogen ion concentrations were the concentration of virus, and the presence of other proteins, amino acids, such as arginine and glycine, and salts.

e. Periodate Ion. Potassium periodate, at an appropriate dilution and in the relative proportion of 8 mg. or more per milliliter of allantoic fluid, acting on LEE virus, destroyed the hemagglutinin (Fazekas de St. Groth and Graham, 1949). Below this concentration hemagglutinin remained, but at concentrations of 1 mg. per milliliter or greater, virus enzyme was damaged and virus would not elute. These results were virtually independent of the final molarity of the periodate.

f. Trypsin. Trypsin did not destroy hemagglutinin, but in high concentration it reduced the enzymatic activity, producing an indicator virus from LEE (Hoyle, 1952; Stone, 1949a).

g. Formaldehyde. Added to allantoic fluid in final concentration of 0.1 to 1.0 %, formaldehyde destroyed the hemagglutinins of PR8 and LEE over a period of several days at 4°C. (Hirst, 1942b; Chu, 1948d). Mumps hemagglutinin was reduced in titer by 0.04 % formaldehyde after 24 hours at

37°C., but not at 4°C., even after 4 weeks in the presence of 0.08 % formaldehyde. A concentration of 0.2 % formaldehyde destroyed 98 % of NDV hemagglutinin after 6 days at 4°C. (Chu, 1948d).

h. Urea. Tyrrell and Horsfall (1954) destroyed the hemagglutinin of PR8 and other strains of influenza with 5 *M* urea acting at 37°C. for several hours.

j. Glycerol. In concentrations of 10 % to 50 %, glycerol protected the hemagglutinin of PR8, NDV, and mumps against deterioration in the presence of 0.1 % formalin at 4, 23, and 37°C. (Cabasso *et al.*, 1951). Fraser (1957) used 50 % glycerol to protect the hemagglutinin of the influenza strain NWS against destruction by heat at 56°C. for 30 minutes. Under these conditions the virus developed the indicator state.

k. Ether. There are considerable discrepancies in the experience of different workers who have treated myxoviruses with ether. Time and temperature are obviously important and there may be strain differences. It is possible that the presence of impurities has been responsible for some of the findings.

Hoyle (1952), using strain DSP (isolated in 1943), produced a small particle hemagglutinin of increased titer but with greatly reduced enzymatic activity. Smith *et al.* (1953) found that PR8 hemagglutinin was destroyed by ether treatment over many hours and could not demonstrate any increase in hemagglutinin even with briefer treatment.

Using pure reagents, both Schafer and Zillig (1954) and Ada and Burnet (unpublished) have obtained hemagglutinin in a form smaller than the elementary body. The latter showed that preparations from several strains were active both as hemagglutinin and enzyme.

m. Alcohol and Alum. The hemagglutinins of PR8, NDV, and other strains may be concentrated and purified by precipitation with methanol or ethanol in the cold under carefully specified conditions (Cox *et al.*, 1947). Alum will also precipitate the hemagglutinin without damage (Bodily *et al.*, 1943).

5. *Effect on Red Cells of Physical and Chemical Agents*

a. Aging. Miller and Stanley (1944) confirmed the finding of Hirst that chick cells gave a lower titer of hemagglutinin as they aged over a period of 4 to 9 days.

b. Heating. Red cells heated at 56 or 65°C. were still able to absorb PR8, and red cell stroma heated at 85°C. for 30 minutes still absorbed nearly half the amount of virus that was absorbed by unheated stroma (Hirst, 1948a).

c. Hydrogen Ion Concentration. Within the range of pH 3.14 to 10.15, red cell receptors were stable for one hour in citrate-phosphate buffer (Hirst, 1948a).

d. Periodate Ion. Hirst (1948a) first stated that concentrations of sodium periodate down to *M*/1000, acting on 0.75 % fowl red cells, destroyed their ability to absorb virus. Various other oxidizing agents were without effect.

He attributed this to an alteration of a polysaccharide, probably the breaking of a bond between adjacent carbon atoms carrying two hydroxyl groups, or one hydroxyl and one amino group.

Later, Fazekas de St. Groth (1949) pointed out that even lower concentrations of periodate, in the range of 2 to 0.2 mg. potassium periodate per gram of red cells, would modify red cell receptors so that virus would adsorb but not elute. The reaction of periodate with red cell receptors and mucoids has thrown considerable light on the chemistry of these substances and has provided valuable biological tools in virus research.

e. Formaldehyde. Red cells treated with formaldehyde in concentrations up to 18 % still absorbed influenza hemagglutinin (Flick, 1948). NDV agglutinated red cells made up as a 2 % suspension in 0.2 % formaldehyde (Burnet *et al.*, 1945).

6. *Increase in Titer of Hemagglutinin at 37°C. in the Presence of Cells*

This phenomenon has been observed with the Victorian strain of Newcastle disease virus on fowl or human cells (Anderson, 1947a) and with Enders's strain of mumps on human cells (Lind, 1948). It is demonstrated as follows: If doubling dilutions of virus are made in normal saline in glass tubes, and fowl or human red cells are added, an end point can be determined by the pattern produced at 37°C. within 30 minutes. If the titration tubes are shaken and again allowed to settle, the end point is 2- to 4-fold the previous reading; a further increase appears after a second shaking and settling and the whole increase may finally reach a total of 8-fold. NDV remaining in the supernatant after absorption with red cells at 4°C. may show an increase in hemagglutinin end point of up to 40-fold on incubation at 37°C. with fresh cells.

It was first suggested that disaggregation of clumps of hemagglutinating units might explain this behavior (Anderson, 1947a), but photographs of virus prepared for the electron microscope do not support this view (Bang, 1948; Cunha *et al.*, 1947; Elford *et al.*, 1948). Burnet (1950) suggested instead that the increase in titer might be associated with the development of agglutinin on stable cells and the increased adsorptive power of particles already firmly attached to one red cell.

C. Elution

1. *Production of Stabilized Cells*

Viruses of the myxovirus group which have adsorbed to red cells will elute at 37°C. over a period of minutes or hours. Elution is due to a reaction between an enzyme on the virus particle and mucoid substrate groupings on the red cell surface. The chemical nature of the substrate is discussed in

Chapter IV by Gottschalk. For the purposes of this chapter, it will be convenient to use the term mucoid as a general one for those components of cells or inhibitors which serve as substrates for the influenza virus enzyme, neuraminidase (Gottschalk, 1957), or the receptor-destroying enzyme (RDE) of *Vibrio cholerae.*

After complete elution of the virus, the red cells are no longer agglutinated by the eluting virus and are said to be stable. The eluting virus will still firmly agglutinate fresh red cells and elute from them in turn. The alteration produced in the red cell is termed receptor destruction.

Elution, or receptor destruction, has a temperature coefficient, being negligible at 0°C. and rapid at 37°C. (Stone, 1949b). Cations are necessary for elution, calcium being more efficient than sodium (Burnet and Edney, 1952). The elution of influenza proceeds best in the presence of 0.1 % calcium and at a reduced rate in the presence of sodium hexametaphosphate. Fluoride, phosphate, and certain resins which remove calcium do not prevent elution (Porterfield, 1952). Elution of NDV proceeds best at pH 6.8 to 7.7 (Sagik and Levine, 1957).

The speed and effectiveness of stabilization varies between different viruses; with many influenza strains it is rare to obtain cells stable enough for experimental work by simple treatment with virus. In these cases it is possible to stabilize the cells by the subsequent addition of the minimal amount of specific immune serum (Burnet *et al.*, 1945).

2. *Properties of Stabilized Cells*

a. Receptor Gradient. Cells stabilized by a virus are not agglutinated by fresh supplies of the same strain (Hirst, 1942b; Burnet *et al.*, 1945). They may, however, be agglutinated by some other strains of myxovirus.

Any series of strains can be arranged in a linear order, called a receptor gradient (Burnet *et al.*, 1946), so that when red cells are stabilized by one strain they are not susceptible to agglutination by any virus earlier in the gradient but are agglutinated by every strain later in the gradient. The order of several strains is mumps, NDV, then the influenza viruses, MEL, WS, LEE, BEL, MIL, B, and swine. The order is the same for human and fowl cells, but chick embryo cells stabilized by NDV are not agglutinated by certain influenza viruses, including MEL and WS (Burnet *et al.*, 1945).

Burnet and his colleagues originally described this gradient, using in certain cases cells stabilized by the combined action of virus for a standard time, and then immune serum. Hirst (1950) found that certain influenza viruses, if allowed to act to completion on the cells, would produce cells insusceptible to all other viruses of the group. No gradient was then demonstrable. Both authors seem agreed, however, that by partial treatment of red cells with

viruses or with the bacterial receptor-destroying enzyme of *V. cholerae*, a gradient can be demonstrated.

b. Altered Electrophoretic Mobility. After stabilization of cells by virus, the electrophoretic mobility of the cell is reduced to a figure which is characteristic of the virus used. The extent of reduction of electrophoretic mobility in general correlates with the position of the strain in the receptor gradient. The mobility of human cells is normally 1.3 μ/sec./volt/cm.; cells stabilized by mumps have a value of 1.18; MEL, 0.80; BEL, 0.75; PR8, 0.74; LEE, 0.74; MIL B, 0.67; NDV, 0.50; and swine, 0.37.

The effect of swine and NDV on electrophoretic mobility is unexpectedly great; the mobility has become lower than expected from their place in the gradient (Ada and Stone, 1950). On the basis of this and other observations, Stone and Ada (1950) suggested that NDV and swine both have two distinct actions on the red cell. One action determines their place in the gradient: both actions contribute to reduction in electrophoretic mobility of the cell. They also believe that the receptor-destroying enzyme (RDE) of *V. cholerae* has the same two actions on red cells. They believe both these actions are mediated by an enzyme. If this is accepted, it follows that each of these viruses possesses two distinct enzymes, or more probably that there are two or more substrates available to a single virus enzyme.

c. The Development of New Antigens on the Red Cell Surface. Following Thomsen's recognition (1927) of the panagglutinability of human red cell suspensions contaminated by certain bacteria, Friedenreich (1928) treated human red cells with diphtheroids and vibrios, and found that the cells become agglutinable by any normal serum. The responsible serum component was called a T agglutinin. Similarly, following treatment of red cells by myxoviruses or RDE, the cells developed T agglutinogens. The T agglutinin titer estimated in serum varied according to the strain of virus used to treat the cells, being greater with viruses further down the gradient. With swine-treated fowl cells, the titer was about 1 : 160 (Burnet *et al.*, 1946).

Burnet and Anderson (1947) demonstrated that human or guinea pig cells treated with RDE of *V. cholerae* and injected into rabbits stimulated the production of new red cell agglutinins to high titer. These new agglutinins reacted with both normal red cells and cells treated with either RDE or viruses of the myxovirus group. The agglutinins for treated cells were not absorbed from the serum by normal cells. Stewart and Quilligan (1951) found a comparable antigenic modification of red cell surface by PR8 and LEE.

d. Special Characters of Cells Treated with NDV or Mumps Virus. Newcastle disease virus and mumps virus agglutinate red cells in normal fashion but have several additional effects not seen with influenza viruses. Their capacity to show an extension of hemagglutinin titer when kept in contact with cells at 37°C. has already been mentioned. When red cells are treated

with relatively large amounts of NDV at 37°C., they rapidly stabilize and are found to have lost all power to absorb or be agglutinated by NDV or mumps virus. They have, however, two new qualities—they agglutinate fresh red cells and they are agglutinated by specific antiviral serum and by a proportion of sera from human cases of infectious mononucleosis (Anderson, 1947a; Burnet and Anderson, 1946). Both these properties are resistant to treatment with RDE and appear to be due to an irreversible union of a proportion of the virus particles with the cell surface.

In support of this view, the following points may be cited:(1) the reaction does not occur with cells treated with RDE before being exposed to virus; (2) treated cells will not only agglutinate fresh cells but will progressively destroy receptors on these; (3) Burnet and Mitchell found that cells showing the typical reactions after treatment with NDV could be prepared for electron microscopy and then showed the presence of typical virus particles on the cell surface.

Mumps virus behaves similarly but the reactions are more difficult to demonstrate than with NDV (Burnet, 1946; Lind, 1948). Mumps and NDV, again unlike the influenza viruses, are hemolytic (Morgan *et al.*, 1948; Kilham, 1949). The conditions for hemolysis are complex, but one feature noted with NDV is that the reaction only takes place at temperatures above 24°C. (human cells) or 28°C. (fowl cells) (Burnet, 1950). Hemolytic action is more readily inhibited or destroyed by heat and ultraviolet irradiation than is hemagglutinin (Chu and Morgan, 1950b), and Gardner and Morgan (1952) found that the hemagglutinin and hemolysin of mumps were differently affected by certain inhibitors of enzymes. The same cell receptors, however, seem to be necessary, and all authors agree that the hemolysin is, or is carried by, the virus particle itself (Beveridge and Lind, 1946; Morgan *et al.*, 1948).

From what has been said, it will be seen that NDV, and possibly mumps, have two groups of actions on red cells. The first corresponds to hemagglutination and stabilization by active influenza viruses; the responsible agents are stable to 56°C., and act at all temperatures in the range 4 to 37°C.

The second group comprises production of agglutinin on stable cells, cell sensitization, increase of hemagglutinin titer at 37°C. on repeated shaking, hemolysis, and an undue reduction of electrophoretic mobility. These actions may or may not depend on one mechanism. The agent or agents in virus responsible for hemolysis are destroyed at 54°C. and act on red cells only above 28°C. (fowl cells) or 24°C. (human cells). We may, for convenience, denote this group of actions as "binding" actions, in contrast to elution, and perhaps compare them with the "binding" action of indicator viruses on red cells at 37°C. (Section 1, E).

D. Enzymes with Receptor-Destroying Properties

In view of the work of Gottschalk and others (see Chapter IV) in characterizing the substrate and mode of action of the enzyme (a neuraminidase) of the influenza virus, only a brief account will be given of some implications of these enzymes for hemagglutinin reactions.

The first enzyme of bacterial origin to be studied was obtained from *Clostridium welchii* by McCrea (1947), but he did not investigate this organism further after the receptor-destroying enzyme was recognized as a product of the growth of *V. cholerae* (Burnet *et al.*, 1946). This has since become a standard reagent for a wide variety of experimental studies with influenza viruses.

1. *The Receptor-Destroying Enzyme (RDE) of Vibrio cholerae*

a. Biological Properties of the Enzyme. The methods of production and purification of this enzyme are still based on those described by Burnet and Stone (1947), but Ada and French (1957) have recently recommended an improved method involving growth in a medium containing neuraminic acid compounds derived from bovine colostrum. In an earlier paper, they described a useful method for its partial purification (Ada and French, 1950).

The enzyme is completely destroyed in 30 minutes at 52.5°C. in the absence of calcium. In the presence of $M/100$ calcium at pH 5.6 to 8.5, the thermal death point is about 60°C. RDE is destroyed by crystalline trypsin at pH 7.2 to 8.5 and it is possible to produce a specific antibody to it (Stone, 1949a; Burnet, 1949a).

RDE adsorbs to red cells, alters receptors to a greater degree than any influenza virus, and elutes. These actions require calcium and occur best at pH 6.8 (Burnet, 1948b, Briody, 1948).

The enzyme does not agglutinate red cells, but when cells are saturated with RDE in the cold, the enzyme will block the adsorption of influenza virus, presumably independently of its enzymatic action, which is minimal at that temperature (Stone, 1947).

The role of calcium is interesting. At 37°C. in the presence of calcium, nearly 97 % of RDE in solution adsorbed to red cells and eluted rapidly. Receptor destruction covered the whole range of the receptor gradient. At 0°C. in the absence of calcium, equilibrium was reached with only about 55 % of RDE on red cells. This system was then brought to 37°C. and receptor destruction proceeded to a point at which only receptors for NDV had been destroyed; at that point all RDE eluted from the cells (Edney, 1949). The amount of calcium which allows the enzyme to develop full titer is in the range 0.2 mM to 0.5 mM but depends on the anion concentration of the medium (Edney, 1949; Porterfield, 1952).

Although full treatment by RDE removes all receptors from red cells, it is possible to halt the action at any stage by the addition of calcium deionizing agents and so to duplicate the action of any myxovirus strain in producing cells at the corresponding place in the gradient. The gradient so produced corresponds to that produced by viruses, with one exception; the mumps virus receptor falls later in the RDE gradient (Burnet *et al.*, 1946). With horse or ox cells the mumps receptor remains intact, even when all influenza receptors have been destroyed (Stone, 1947).

Electrophoretic mobility of guinea pig cells acted upon by RDE *in vivo* fell from 1.10 to 0.20 μ/sec./volt/cm., which is a lower figure than produced by any influenza virus (French and Ada, 1954). The electrophoretic mobility of human cells fully treated *in vitro* by RDE falls from 1.30 to 0.17 μ/sec./volt/cm. (Ada and Stone, 1950).

RDE-treated cells develop T agglutinogens, and we have discussed above the development of other new surface antigens.

It is evident that RDE duplicates the action of the virus enzyme but takes the reaction further than any known myxovirus. As might therefore be expected, RDE will remove agglutinating virus from red cells more rapidly than the virus itself would normally elute, and will also remove indicator viruses.

b. Periodate-Virus-RDE (PVR) Cells. Fazekas de St. Groth (1949) showed that treatment of fowl or human red cells with amounts of potassium periodate (KIO_4) in the range 0.2 to 2.0 mg. KIO_4 per gram of cells produced a modification of cell receptors, rendering them insusceptible to destruction by virus enzyme and hence preventing elution of adsorbed virus. If, after adsorption of virus with agglutination, the cells are treated with RDE, receptors unoccupied by virus are destroyed so that the cells are stabilized but carry firmly attached virus particles. Such PVR cells have properties analogous to normal cells on which NDV or mumps virus is irreversibly bound.

E. Indicator Virus

Francis (1947) found that if influenza B virus LEE was heated to 56°C., its hemagglutinating activity remained almost intact, but the heated virus was not only more sensitive to the hemagglutinin-inhibiting action of immune serum than active virus but was also inhibited by normal serum. This action of normal serum was subsequently shown by Anderson (1948) and McCrea (1948) to be due to a mucoid inhibitor. The change in the virus following such mild heat treatment is usually referred to as conversion to indicator virus. Stone (1949b) used the term because viruses in this state indicate the presence of inhibitory mucoids in various animal fluids and extracts and can be used to titrate them.

The most suitable method of preparing indicator virus varies from strain to strain. Francis's original method of heating allantoic fluid at 56°C. for 30 minutes is satisfactory for LEE and many other strains. Others, such as MEL, are not changed to the indicator state by this treatment. Stone (1949b) developed the method of heating allantoic fluid adjusted to pH 8.5 with citrate or some other calcium deionizing agent added. In the same paper, Stone describes a reversible indicator state which she induced in one strain of PR8 and WSE. A variety of other methods may produce similar changes with certain strains; these include treatment with periodate ion and trypsin (Hoyle, 1952; Stone, 1949a).

Indicator viruses agglutinate red cells but do not elute; in general, they occupy a position in the receptor gradient a little below the position occupied by the active virus; they can be removed from red cells by the action of RDE or of an active virus lower in the gradient. They have no enzymatic action on soluble mucoid inhibitors; Hirst (1948b) and Burnet (1948b) have suggested that indicator virus is inhibited by mucoid because it cannot destroy the mucoid receptors. Anderson *et al.* (1948) and Stone (1949b) pointed out that this simple explanation may not be complete or correct. For example, at 0°C., where enzyme action is negligible, active virus should be inhibited by mucoids, but this is not the case. Smith and Westwood (1950) have also argued that loss of enzyme does not, *ipso facto*, make a virus an indicator virus.

Active virus reacts with red cells, changing the receptors and completely eluting. Indicator viruses, at least of LEE and MEL, do not elute, but have a second reaction with fowl red cells leading to a firm union of a proportion of the virus particles with the red cells, a union which cannot be broken by RDE. This reaction requires 15 to 30 minutes at 37°C., or a longer time at lower temperatures. It does not occur with human cells (Burnet, 1952b).

F. Inhibitors of Hemagglutination

1. *Specific Antibody*

Specific antihemagglutinating antibody appears following infection or vaccination with most influenza strains. Its specificity corresponds approximately to that of active immunity, but there is still much uncertainty about the details of cross-reactions among strains and antisera. The most recent discussions are by Jensen and collaborators (1957; Jensen and Petersen, 1957; Jensen, 1957). The antihemagglutinin response of a human depends largely on the serological character of his first experience of influenza (Davenport and Hennessy, 1956).

Specific antibody is stable at 62°C. for 30 minutes. It is not destroyed by incubation with RDE, influenza virus, or moderate concentrations of periodate ion.

Union between antibody and virus is reversible during a short period after mixing (Isaacs, 1948; Burnet *et al.*, 1945). Antihemagglutinin titers of specific antibody depend a little on the source of the fowl cells used, but this effect is not so pronounced as in the case of Chu inhibitor (Stuart-Harris, 1943; Hirst, 1943b).

2. *Chu Inhibitor*

This term is used to denote nonspecific thermolabile factor(s) in serum, inhibiting viral hemagglutinin and sometimes infectivity.

Hirst (1942a, 1943a) and Burnet and McCrea (1946) described a factor in normal rabbit and ferret serum and in ground ferret lung which inhibited hemagglutination by fresh virus. Similar nonspecific inhibitors have been found in other tissues of humans, animals, and chick embryos. Beveridge and Lind (1946) found in allantoic fluid and yolk sac suspensions a nonspecific inhibitor of mumps virus. This hemagglutinin is also inhibited by the polysaccharide of Friedlander bacillus type B (Ginsberg *et al.*, 1948).

a. Relation to Virus. The union between virus and Chu inhibitor is reversible and the titer of inhibitor depends on the affinity between cells and virus. Thus, the titer of inhibitor against any one strain of virus varies with the source of fowl cells, being greatest with those fowl cells having the least affinity for virus (Anderson *et al.*, 1946). Probably for similar reasons Chu inhibitor is more inhibitory against recently isolated strains than against classic egg-adapted viruses (Tamm, 1954b; Chu, 1951).

Chu inhibitor may also prevent infection in certain circumstances. Burnet and McCrea (1946) found that normal ferret sera would prevent infection of the amniotic cavity of chick embryos by BEL virus and protected mice against inoculation with BEL D virus. Chu (1951) described a thermolabile inhibitor in mouse serum which prevented infection of mice by unadapted but not by adapted strains of influenza. This may be the same as the inhibitor described by Ginsberg and Horsfall (1949) in sera of humans, guinea pigs, mice, and rabbits, which, however, was labile on storage at 4°C. for several weeks. Active virus does not destroy Chu inhibitor (Chu, 1951).

b. Physical and Chemical Properties. A definitive characteristic of this inhibitor is its destruction by heat. The temperature of destruction varies a little with the source of inhibitor but is in the range of 56 to 62°C. (Burnet and McCrea, 1946; McCrea, 1946).

McCrea (1946) precipitated this type of inhibitor from rabbit serum by 33 % saturation with ammonium sulfate. He believed the agent to be a gamma globulin.

Anderson (1948) was able to destroy this inhibitor by treatment of normal human serum with preparations of crude RDE. Mulder and van der Veen (1948) applied this technique to ferret sera, using crude RDE. Chu and

associates (1950a) and Chu (1951) believed that the RDE must be used in the crude state and Appleby and Stuart-Harris (1950) supported this, reporting that purified preparations were inactive. Tyrrell and Horsfall (1952) confirmed the action of crude RDE.

Against this background, Sampaio and Isaacs (1953), working with rabbit sera, decided the active agent in crude RDE was the trypsin-like component described by Stone (1949a). Pure trypsin also destroyed Chu inhibitor.

The action of periodate ion on Chu inhibitor has been discussed by various authors. Chu (1951) claimed it did not destroy this inhibitor. McCrea (1948) found both the specific antibody and Chu inhibitor in rabbit serum were reduced only slightly in titer by periodate ion, but Hirst (1948a) had reported a considerable reduction of inhibitor, possibly Chu inhibitor, in rabbit serum.

Burnet and Lind (1954) modified a method of Davoli for the removal of Chu inhibitor by periodate. Large concentrations of periodate reduced specific antibody in rabbit sera, but a concentration of M/150 left the specific antibody intact, while removing all nonspecific inhibitor.

3. *Mucoid Inhibitors*

The development of a precise chemical approach to the mucoid inhibitors of hemagglutination by Gottschalk, Blix, Klenk, and others (see Chapter 4) in recent years has rendered it unnecessary to recapitulate the large amount of work carried out on this theme between 1946 and 1952. Chemical aspects are discussed by Gottschalk in the following chapter; here it is only necessary to outline the main phenomena encountered in the earlier work done with a primarily biological orientation.

a. Sources of Inhibitor. Mucoid inhibitors are found in a wide variety of vertebrate tissues and secretions. Originally described in mammalian sera (McCrea, 1948), they are also present in egg white (ovomucin) (Gottschalk and Lind, 1949), tears (Anderson, 1948), saliva (Francis and Minuse, 1948; Seltsam *et al.*, 1949), urine (Tamm and Horsfall, 1952; Perlmann *et al.*, 1952), and many mucinous secretions. They were found in high concentration in salivary glands of man, ferret, mouse, and sheep, and in lower concentrations in salivary glands of other species (Anderson, 1950; McCrea, 1953). Mucoid inhibitors in various degrees of purity have been recovered from ovomucin, human urine, bovine submaxillary gland (Curtain and Pye, 1955), sheep salivary gland (McCrea, 1953), and human meconium (Curtain *et al.*, 1953).

b. Biological Properties. As originally studied, mucoid inhibitors had little or no action on active virus but strongly inhibited hemagglutination by heated (indicator) virus. The inhibitory power can be destroyed by the action of active virus or RDE (Anderson, 1948). The optimal conditions for the reaction include the presence of calcium ions and a pH of about 6.0.

As with the action between virus or RDE and red cells, so with mucoid inhibitor a clear receptor gradient can be established (Stone, 1949c). An inhibitor treated by an active virus will no longer inhibit the indicator virus of the same strain, but will inhibit indicator viruses further down the gradient. This effect can be duplicated by partial treatment with RDE.

Most mucoid inhibitors are active against all indicator viruses but a few have a more restricted range. For example, bovine submaxillary gland mucoid inhibits only indicator PR8 and strain 1233 (Curtain and Pye, 1955). Sheep salivary mucoid does not inhibit indicator MEL.

Certain mucoid inhibitors inhibit hemagglutinin of both indicator and active virus. One example is the urinary mucoprotein, which inhibits active MEL and WSE (Burnet, 1952c). Treatment of mucoid inhibitors with potassium periodate will destroy inhibiting power but at lower concentrations treatment results in an increased capacity to inhibit hemagglutination by active viruses. Examples are given by Burnet (1948a, 1949b).

Burnet (1952a) has suggested the generalization that, whenever an inhibitor is effective against hemagglutination by indicator virus of the same strain, the active virus will destroy the inhibitory action in typical enzymatic fashion. When active virus hemagglutination is also inhibited, it is usual to find little or no enzymatic action.

For purposes of discussion, we have assumed a clear distinction between Chu inhibitor and mucoid inhibitor. McCrea (1948), however, has surmised that the Chu inhibitor, which is probably a globulin, and the mucoid inhibitor are united in solution. It may not be necessary to go as far as Smith and Westwood (1950) in suggesting that there are not distinct qualitative differences between inhibitors of heated and unheated virus.

4. *Other Types of Inhibitor of Hemagglutination*

Ginsberg *et al.* (1948) have described the inhibition of mumps hemagglutinin by the polysaccharide of Friedlander bacillus type B, which will also inhibit mumps infectivity in the egg. This inhibitor acts on the host cell, probably not on the virus, and there is no indication that it is in the class of compounds acted on by RDE. Similarly, the apple pectin and other substances of Green and Woolley (1947) and Woolley (1949) are probably not related to red cell receptor material. We assume the polysaccharide of *Klebsiella aerogenes* and *K. cloacae* also belong outside the virus substrate class (Macpherson *et al.*, 1953), together with the pneumococcal filtrate substance of Svec and Forster (1947).

G. Mechanism of Hemagglutination

It is a reasonable assumption that hemagglutination is due to the mechanical bridging of two or more red cells by virus particles which simultaneously

adsorb to each red cell, although Chesbro and Hedrick (1957) have speculated otherwise.

Two hypotheses have been proposed to explain virus adsorption to the red cell. Both were developed in the absence of knowledge about the nature of the substrate of the virus enzyme and there is still very inadequate knowledge of the detailed structure of either of the two surfaces concerned. With the development of a more rigorous experimental approach, it is likely that a much more satisfactory interpretation in physical terms will be available for the physical aspects of the hemagglutinating process. For the present, however, both theories will be described briefly, and an explanation will be attempted, in terms of each theory, of certain facts of the relationship between virus and red cell. The truth may well be a compromise between these two theories.

1. *Hypothesis of Physical Adsorption*

This hypothesis distinguishes clearly between adsorption of virus and subsequent enzymatic reaction. The red cell receptor is considered to be an area considerably larger than the virus particle, and to contain a large number of mucoid substrate groupings. The active groups of the virus enzyme might form only a limited portion of the virus surface (Anderson, 1947b).

Adsorption is considered to be a function of all of the virus surface which is opposed to the red cell, and also of a comparable portion of the receptor area, including both substrate groupings and other red cell structures not susceptible to alteration by virus enzyme.

Adsorption of influenza virus would correspond with the adsorption onto red cells of other viruses, such as Japanese B encephalitis and GD VII, where no virus enzyme enters into consideration. Myxoviruses would differ only in the fact of subsequent elution through receptor destruction.

In support of this hypothesis is the ready removal of influenza virus from red cells, without receptor destruction, by raising the ionic level of the medium (Hirst, 1949). There is also the finding of Tamm (1954a) of reversible union of LEE virus with cat erythrocytes. Virus can be recovered from cat erythrocytes, again without receptor destruction, by raising the temperature from 5 to 24°C. in a suitable ionic medium.

It is assumed that, after adsorption, a virus becomes orientated so that the enzyme reacts with mucoid substrate in the vicinity; and that each time one substrate grouping is enzymatically altered the local attractive forces between red cell and virus are diminished. When a sufficient proportion of groupings in the vicinity has been altered, the virus particle is believed to roll or slide to an adjacent portion of the receptor area or to a nearby receptor area. This phenomenon has been termed "browsing." This "sufficient proportion of groupings" in any one area is a characteristic of the virus strain; it depends

on the structure of the whole virus surface, and determines the place of the virus in the receptor gradient.

When all the red cell receptor areas have been browsed over in this way, the virus must leave the red cell. It is possible for a virus with greater affinity for red cells—one further down the gradient—to adsorb to these treated red cells, to alter a proportion of the remaining substrate groupings, and to elute in its own turn. On this basis the receptor gradient is explained.

As an alternative to the idea of browsing, Sagik *et al.* (1954) have proposed that a virus on the red cell may "act at a distance" in a fashion similar to that demonstrated for phage on bacteria.

Indicator virus possesses no active enzyme and does not elute from a suspension of red cells, but fresh virus or RDE added to the suspension will remove the indicator virus. This would be due to the alteration by the added enzyme of the necessary proportion of substrate groupings adjacent to the attached indicator virus. It seems necessary to assume that the production of the indicator state alters the virus surface to give it a relatively greater affinity for soluble mucoid inhibitor than for red cells (Anderson *et al.*, 1948).

It is readily agreed that since periodate alters substrate groupings, periodate-treated receptors will not permit virus elution through enzymatic action.

2. *Hypothesis of Enzyme Substrate Attraction*

This would regard union of virus and receptor as due primarily to union between the functional group of the virus-enzyme and the substrate groups in the receptor mucoid and would regard the part played by other components of the two surfaces as being concerned only in determining the mutual accessibility of virus enzyme and cell substrate groups (Hirst, 1942b; Burnet, 1948c, 1951b; Burnet and Lind, 1950; Chu, 1948d).

Explanations of browsing and elution would follow as before, but the receptor gradient is interpreted in terms of receptors on the red cell which are of varying accessibility to different viruses (Burnet *et al.*, 1945).

The enzyme on indicator virus is claimed to be so modified as to be incapable of elution but still to retain the pattern needed for it to unite with substrate groupings on the red cell. It is difficult, on this basis, to explain the ready removal of indicator virus by RDE or live virus. Receptor mucoid, partly altered by potassium periodate, would similarly bind virus enzyme but not allow completion of enzymatic action or elution.

In nearly every case, the development of an indicator state in treated virus is strictly correlated with loss of enzyme activity of the virus on the inhibitor concerned. This has been advanced in support of the hypothesis of enzyme substrate attraction as the mechanism of hemagglutination, but it also appears compatible with the first hypothesis described above.

II. Particulate Hemagglutinin with no Eluting Enzyme

A. Arthropod-borne Encephalitides

1. *Historical*

In 1936, Burnet found that chick embryos inoculated on the chorioallantois with louping ill virus and showing visceral lesions regularly contained virus attached to the red blood cells. This seems to be the first indication of the capacity of a member of the arthropod-borne virus group to attach to erythrocytes.

Hemagglutination by this group of viruses was first described by Sabin and Buescher (1950) with Japanese B, West Nile, and far eastern encephalitis viruses. Sabin (1951) later added St. Louis and possibly western equine encephalitis, and Sweet *et al.* (1953) dengue 1 and dengue 2. Macdonald (1952) described hemagglutination by Murray Valley encephalitis. Casals and Brown (1953) confirmed West Nile, dengue 1, dengue 2, and Japanese B, and added Ilheus, Ntaya, and yellow fever (Asibi). Clarke and Theiler (1955) found Semliki Forest and Bunyamwera agglutinins in mouse serum. Some neurotropic viruses are reported to lose their hemagglutinin after many mouse passages (Sabin, 1951).

2. *Cells Agglutinated*

Japanese B virus is typical of these viruses in agglutinating red cells from day-old chickens, but generally not from adult fowls, nor from humans nor rhesus monkeys (Sabin and Buescher, 1950). Both Murray Valley encephalitis virus and Japanese B virus agglutinate red cells from adult pigeons (Macdonald, 1952). Russian far eastern encephalitis agglutinates sheep cells at pH 7.5, West Nile agglutinates baby chicken and sheep cells at pH 7.3, and St. Louis virus agglutinates baby chicken cells at pH 6.5 to 7.0 (Sabin, 1951).

3. *Preparation of Hemagglutinin*

The hemagglutinin may be extracted from 21-day-old (Sabin and Buescher, 1950) or suckling mouse brains (Macdonald, 1952; Casals and Brown, 1953) at appropriate hydrogen ion and salt concentrations. Physiologically normal saline will extract Japanese B and Murray Valley hemagglutinins, which should then be centrifuged at 20,000 *g* for 60 minutes, and may be stored for long periods at 4°C. in the liquid state, or preferably lyophilized or frozen at — 20°C. (Casals and Brown, 1954). In the liquid state, at 5°C. the hemagglutinin increased in titer over a period of 3 to 10 days (Sabin and Buescher, 1950). No hemagglutinin has so far been found in extracts of virus infected chorioallantoic membrane.

4. *Hydrogen Ion Concentration*

In general, hemagglutination occurs best at 4°C. and pH 7.0 with members of group B, and at 37°C. and pH 5.6 to 6.4 with members of group A (Casals and Brown, 1954); but there are large individual variations as, for example, described for western equine (Chanock and Sabin, 1954*a*), West Nile (Chanock and Sabin, 1954b), dengue (Sweet and Sabin, 1954), and St. Louis encephalitis viruses (Chanock and Sabin, 1953).

Some hemagglutinins are unstable at the pH necessary to demonstrate hemagglutination. It is then necessary to so buffer the cell suspension that the pH of the virus suspension is adjusted suitably on addition of red celsl (Sabin, 1951).

5. *Relation of Hemagglutinin to Virus Particle*

It is generally assumed that the hemagglutinin is associated with the virus particle. Sabin was able to deposit the hemagglutinin by centrifugation at 58,300 *g* (Sabin and Buescher, 1950), so that it is probably at least the size of the infectious particle.

No enzyme has been found associated with the hemagglutinin, and the receptor-destroying enzyme of *V. cholerae* does not prevent adsorption of the arthropod-borne viruses to red cells (Sabin and Buescher, 1950).

6. *Nonspecific Inhibition of Hemagglutinin*

Sera of normal humans, rabbits, monkeys, and mice contain an inhibitor of the hemagglutinin. In human sera the inhibitor is active to a titer of about 1 : 1000 against 10 agglutinating doses of Japanese B virus (Sabin and Buescher, 1950).

Nearly all of the inhibitor was removed by extraction with chloroform, benzine, acetone, or ether (Sabin, 1951), by Seitz filtration (Casals and Brown, 1954), or absorption with the clay Bentonite (Oker-Blom *et al.*, 1950; Clarke and Casals, 1955). Dialysis, filtration through sintered glass, or through collodion membranes of APD, 303 mμ, did not remove inhibitor, but membranes of APD, 69mμ, retained part of the inhibitor from native serum, and all the inhibitor from heated serum (Casals and Brown, 1954). Acetone extraction is favored for the routine removal of inhibitor in practice, especially with certain animal sera.

The inhibitor was relatively stable to boiling and it was not affected by proteinase or potassium periodate; but the lecithinase of *Clostridium perfringens* in the presence of calcium rendered it susceptible to heat inactivation (Sabin, 1951 ; Sabin and Buescher, 1950).

What is possibly a similar inhibitor is present in saline extracts of mouse brain, from which it can be sedimented at 20,000 *g* for 60 minutes. If not

removed from virus preparations by centrifugation, it inactivates the hemagglutinin at 5°C. in 3 to 4 days (Sabin and Buescher, 1950). Japanese B hemagglutinin was inhibited also by boiled extracts of *Clostridium histolyticum* and *Cl. lentoputrescens* even in dilutions of 10^{-6}, by 1 : 2000 diethyldithiocarbamate in buffer, and also by zinc sulfate in concentrations of 0·03 to 1.92 μg. per unit of hemagglutinin. Iron, manganese, magnesium, mercury, and copper were not inhibitory (Sabin and Buescher, 1950).

7. *Serological Grouping*

The hemagglutinins have been divided serologically into three groups and it is possible that more groups will be defined later (Casals, 1957; Casals and Brown, 1954). There are cross-reactions between members of any one group but these reactions are slight or absent between groups. Group A includes western, eastern, and Venezuelan equine encephalitis, Semliki Forest, Sindbis, and Mayaro viruses; Group B includes Japanese B, St. Louis, Murray Valley, West Nile, yellow fever, dengue 1, dengue 2, Ilheus, Ntaya, Russian far eastern, Uganda S, and Zika viruses.

B. Enteroviruses

1. *GD VII*

a. Hemagglutinin. The GD VII strain of Theiler's virus was described by Theiler and Gard (1940). Lahelle and Horsfall (1949) noted the hemagglutination of human group O cells at 4°C., but not at 23 or 37°C. The virus eluted at 37°C. Hemagglutination occurred in the pH range 4.3 to 8.3. The virus did not agglutinate cells of monkey, horse, sheep, cat, dog, guinea pig, hamster, mouse, or fowl.

GD VII adsorbed to human cells at 20°C. but did not agglutinate them, or agglutinated them only poorly. However, after treatment of the virus by trypsin, it hemagglutinated at 20°C. (Morris, 1952). Trypsin-treated virus occasionally agglutinated red cells from monkey and guinea pig at 4°C.

Virus on human red cells eluted at 37°C. without damage to the cells, and again agglutinated the cells on cooling to 4°C. (Fastier, 1950). The receptors involved differed from those to which influenza attached, and periodate ion and RDE of *V. cholerae* did not affect GD VII hemagglutination.

GD VII adsorbed to red cells in a salt-free medium at 4 or 18°C., but did not hemagglutinate at 4°C. unless salts were present to a concentration of at least 0.025 *M* (Fastier, 1951b). Elution into an electrolyte-containing medium was retarded by calcium ions; Lahelle and Ward (1951) found elution to occur into a buffered glucose medium at 4°C.

The hemagglutinin of GD VII was reduced to 1 % of its initial titer by heating to 56°C. for 30 minutes, but stored well at 4°C. for over a month (Lahelle and Horsfall, 1949).

The infective virus followed the hemagglutinin onto and off red cells and the ratios of hemagglutinin to infectivity were fairly constant at different stages of infection of the mouse. For these reasons it is believed that hemagglutination is a function of the infective virus particle (Lahelle and Ward, 1951; Morris, 1953).

b. Nonspecific Inhibition. A nonspecific inhibitor is present in lipid-free extracts from normal mouse brain and other organs (Fastier, 1950, 1951a), and also in normal serum of mice, rats, guinea pigs, and rabbits. It can be partly inactivated by trypsin (Morris, 1952). From human urine and chick allantoic fluid a fairly pure inhibitor can be isolated which is heat stable, and combines with the virus at 4°C. and elutes from it at 22°C. (Tamm and Tyrrell, 1954). It is electrophoretically distinct from the mucoid inhibitor of influenza.

A mucopolysaccharide inhibitor of hemagglutinin and infectivity was recovered from intestinal tissues of adult mice by Mandel and Racker (1953b). Union between inhibitor and virus is probably determined by weak electrostatic forces and can be broken by reducing the concentration of electrolytes. Both components—inhibitor and virus—are then recovered in an active form (Mandel, 1957). There is a fecal enzyme of mice which destroys this inhibitor (Mandel and Racker, 1953a).

2. *Other Strains*

Olitsky and Yager (1949) and Hallauer (1949) described the agglutination of sheep cells at 4°C. by the EMC group of viruses—Columbia SK, Columbia MM, encephalomyocarditis and Mengo encephalitis. They elute at 20°C., like GD VII (Gard and Heller, 1951). Verlinde and de Baan (1949) stated that the cells can be rendered inagglutinable by prior treatment with RDE of *V. cholerae.*

Goldfield *et al.* (1957) recently reported that some ECHO * viruses and Coxsackie B3 agglutinate washed group O human cells at room temperature. The preliminary data suggest that the hemagglutinin is associated with the infectious particle. In this, the human viruses resemble the murine enteric virus, GD VII, and the EMC group.

RDE has no demonstrable action on ECHO receptors, and apparently receptors for ECHO viruses differ from those for myxoviruses. Further studies in this field should be of very great interest.

C. Pneumonia Virus of Mice (PVM)

Pneumonia virus of mice (PVM) is generally latent in mouse lung, but may produce clinical disease (Horsfall and Hahn, 1940). It may be extracted in saline, and in such a preparation is combined with tissue elements from the

* Enteric cytopathogenic human orphan.

lung. These can be removed by heating at 70 to 80°C. for 5 to 30 minutes (Mills and Dochez, 1944), or by treatment with alkali (Curnen and Horsfall, 1947). The hemagglutinin so liberated is about 40 mμ in diameter (Curnen *et al.* 1947). There is no associated enzyme.

The hemagglutinin, which is probably identical with the virus particle, agglutinates red cells of mouse and hamster but not of human, cat, dog, sheep, ferret, rat, guinea pig, or fowl (Mills and Dochez, 1945).

III. Hemagglutinin Separate from the Virus Particle

A. Psittacosis Group

Mouse meningopneumonitis virus grown in the chick allantoic cavity agglutinated mouse erythrocytes, but not red blood cells from 11 other species tested (Hilleman *et al.*, 1951).

Agglutination occurred at pH 7.0, at 24 or 37°C., but not at 4°C.; it was inhibited by specific antibody. The hemagglutinin was also inhibited by calcium ions in concentrations as low as 0.00078 *M* and by 0.005 *M* magnesium, and therefore was not active in undiluted allantoic fluid.

The hemagglutinin was smaller than the infectious particles, which could be removed by centrifugation at 13,000 r.p.m. About half the hemagglutinin was sedimented at 18,000 r.p.m.

A similar hemagglutinin is formed in the chick allantoic cavity by the feline pneumonitis virus of Baker (Gogolak, 1954) and by psittacosis.

The psittacosis hemagglutinin is a complex of lecithin and nucleoprotein, both of which are also present in the infective virus particle (Gogolak and Ross, 1955). These authors believed that the hemagglutinin was either incomplete virus material not incorporated in the elementary body, or a degradation product of the virus; they showed that nonhemagglutinating dead virus particles would stimulate the formation by roosters of antibody specific for the hemagglutinin. They demonstrated similar antigenic factors in the hemagglutinin and the virus elementary body.

The biological behavior of the hemagglutinin closely resembled that described for mouse meningopneumonitis virus and the two hemagglutinations were serologically identical.

B. Poxviruses

Vaccinia hemagglutinin was first described by Nagler (1942). After several hints that the virus produced two hemagglutinins (Chu, 1948a,b; Burnet and Stone, 1946), Gillen and associates (1950) separated the two by centrifugation of chorioallantoic membrane extract at 17,000 r.p.m. in a Sorval head. In native membrane extracts the two are apparently linked and behave as a

single hemagglutinin, separate from the virus particle. Filtered together, both are retained by a Seitz disc; but filtered in the separate state, the heavier hemagglutinin is retained by the disc, while the lighter hemagglutinin is filterable. The lighter hemagglutinin was destroyed at 56°C. after 45 minutes, and agglutinated red cells at all temperatures from 5 to 37°C. The heavier hemagglutinin survived heating at 70°C., and while it reacted with red cells to lower titer at 5°C., hemagglutinated best at 37°C., as did the combined native hemagglutinin (Clark and Nagler, 1943).

Previous studies had considered these hemagglutinins as one agent. They had found the titer to vary considerably over a range of fowl red cells, some fowl cells being completely insusceptible (Burnet and Stone, 1946; Burnet and Boake, 1946) and only about 50 % of fowl cells giving a good titer (Clark and Nagler, 1943). The proportion of fowls having easily agglutinable red cells increases with age, from 0 % of embryos or chicks 2 days old to 23 % of chicks 14 days old and 55 % of fowls 6 to 30 months old (Clark and Nagler, 1943). Cells from a proportion of pigeons are also agglutinated (Burnet and Stone, 1946) but not cells from man, horse, sheep, rabbit, finch, or canary. Mouse cells are occasionally agglutinated to low titer by vaccinia. Ectromelia (mousepox) agglutinates all mouse cells, and also those fowl cells sensitive to vaccinia. No enzyme has been found associated with vaccinia hemagglutinin, and the red cell receptor for influenza and vaccinia are distinct.

Vaccinia hemagglutinin is believed to be predominantly phospholipid. It is inactivated in the presence of calcium by the type C lecithinase of *Cl. welchii* α-toxin, and by the type A lecithinase of cobra venom (Stone, 1946a). Those fowl cells sensitive to vaccinia hemagglutinin are also agglutinated by suspensions of tissue lipids (Burnet and Stone, 1946) and suspensions of pure and mixed lipids of the phospholipid group (Stone, 1946b). Whereas vaccinia hemagglutinin is inhibited only by specific antibody, nonspecific lipid agglutinin is inhibited by normal serum.

Vaccinia hemagglutinin has been recovered from chorioallantoic membrane where it develops during the production of infective virus (Metcalf, 1955), from rabbit skin, rabbit testis, and sheep lymph (Chu, 1948a). It was not found in calf lymph by Nagler (1942), possibly because of the presence of an inhibitor.

Two strains of neuro-vaccinia, and two rabbitpox strains, were found by Fenner (1958) to lack any capacity to produce hemagglutinin.

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Chapter IV

Chemistry of Virus Receptors

Alfred Gottschalk
Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Infection of a host cell by a virus particle is initiated by the entry of the virus into the cell. Several mechanisms are known providing such an entry. Phages of the T system infecting *Escherichia coli* B, a bacterium surrounded by a multi-layer membrane consisting mainly of rigid, structural polysaccharides and lipopolysaccharides, have evolved an elaborate mechanism of invasion. Specific adsorption (chemisorption) of the phage to the surface of the susceptible coli strain is followed by enzyme action on membrane constituents. By these events a passage is formed through which the genetic material (DNA) of the phage is introduced into the interior of the host cell. Plant viruses enter their hosts through vector or other lesions of the surface cells of leaves, etc. For most animal viruses it seems to be characteristic that their host cells are endowed with phagocytic powers. Thus, the epithelial cells of the respiratory and alimentary mucous membranes, the alveolar cells of the lung, the vascular endothelial cells, the endothelial Kupffer cells, and mesenchymal cells are known to have phagocytic properties and to be host cells for various viruses, the intake of the virus by the host cell being termed "viropexis" (Fazekas de St. Groth, 1948b). Animal viruses with a wide range of host cells, like vaccinia virus, ectromelia virus, psittacosis virus, and others, rely for propagation on random collisions with a phagocytic host cell, once having entered the body through a lesion of the skin, the bite of a mosquito, or through an internal surface. Animal viruses with a greatly restricted range of host cells, especially viruses infecting the respiratory tract, have developed a specific mechanism of attachment to their host cells as a preliminary to the intake of the virus into the cell. The specificity of this attachment, like the specificity of an enzyme for its substrate or an antibody for its antigen, resides in the complementariness of molecular structures present at the surfaces of the host cell and of the virus particle. The segment of the host cell surface instrumental in binding the virus is called the *cellular receptor*. It is only for the myxovirus group, comprising influenza viruses A, B, C and D, Newcastle disease, mumps, and fowl plague viruses, that the chemistry of the cellular receptors has been elucidated, at least to some extent.

The fundamental discoveries demonstrating the presence of a specific cellular receptor for influenza virus were made by Hirst and by Burnet and co-workers. Hirst (1942a,b) observed that influenza virus at 4°C. was firmly adsorbed to fowl red cells and remained so for 18 hours; by forming bridges between the cells the virus effected hemagglutination. Raising the temperature to 37°C. resulted in spontaneous elution of the virus from the red cells; whereas the latter were rendered inagglutinable, the virus remained functional. Hirst concluded from the results (1) that the virus attaches itself to a receptor substance at the surface of the red cell, and (2) that, by virtue of an enzyme embedded in the virus coat, the receptor is altered in such a way as no longer to bind the original or any fresh influenza virus. Additional and quite independent proof for the existence of a specific receptor substance as binding site for the influenza virus was provided when Burnet and co-workers (1946; Burnet and Stone, 1947) showed that an exo-enzyme, obtained from the culture filtrate of *Vibrio cholerae* and referred to as receptor-destroying enzyme (RDE), rendered erythrocytes inagglutinable. The similarity of the point of attack on cellular receptors of the soluble vibrio enzyme and of the influenza virus particle was proved in several ways. Red cells, pretreated by one of the two agents, failed to adsorb the other one, and adsorption at 0°C. of one agent onto the intact cells blocked the absorption of the other agent. Moreover, erythrocytes were made agglutinable by normal human sera to equivalent titers when treated with influenza virus and with RDE, respectively (Stone, 1947).

Red blood cells are not host cells for influenza virus propagation. However, further research left little doubt that they are most suitable models for the virus-host cell interrelationship. It was found that influenza virus is adsorbed to and elutes spontaneously from the susceptible respiratory cells of the excised and surviving ferret and mouse lungs (Hirst, 1943; Fazekas de St. Groth, 1948a), and that pretreatment with RDE renders the respiratory surface of the excised mouse lung unable to adsorb influenza virus (Fazekas de St. Groth, 1948a). Perhaps the most spectacular demonstration of the essential role played by the cellular receptor in the initiation of virus infection was the experiment in which infection of the respiratory tract of the intact mouse by inhaled influenza virus was prevented by pretreatment with RDE (Stone, 1948).

The problem of specific influenza virus receptors was greatly advanced when a group of natural substances, apparently with a common feature, was shown to inhibit virus hemagglutination. Francis (1947) observed that normal human serum inhibited hemagglutination by influenza virus, provided the virus was previously heated at 56°C. for 30 minutes. Subsequently, a variety of mucoproteins and mucopolysaccharides, some of them prepared in a highly purified state, was found to inhibit hemagglutination by indicator virus.

Indicator virus is defined as a virus pretreated by heat under specified conditions so as to destroy infectivity and enzymatic activity without impairing the hemagglutinin (Briody, 1948; Stone, 1949a). Among the inhibitors were a mucopolysaccharide from erythrocytes (De Burgh *et al.*, 1948; McCrea, 1953a), ovarian cyst mucin (Burnet, 1948), serum mucoprotein (McCrea, 1948), urine mucoprotein (Tamm and Horsfall, 1950), meconium mucoprotein (Curtain *et al.*, 1953), sputum mucoprotein (Marmion *et al.*, 1953), all of human origin, ovomucin of hen egg white (Gottschalk and Lind, 1949a; Eckert *et al.*, 1949), and sheep submaxillary gland mucoprotein (McCrea, 1953b). The inhibitory capacity of these mucoids was lost upon treatment with living (infective) virus or with RDE (Burnet, 1951; Gottschalk, 1954a). Two inferences were drawn from the data presented. First, the coincidence in the effect of the influenza virus particle and the vibrio enzyme on red cell receptors, host cell receptors, and hemagglutinin inhibitory mucoproteins was taken as strong support for Hirst's suggestion of the presence of an enzyme at the surface of the virus. Second, the cellular receptors and the soluble inhibitory mucoproteins were regarded as chemical analogs, their common structural feature competing for and being susceptible to the isodynamic enzymes of influenza virus and *V. cholerae* (Anderson *et al.*, 1948).

These results and their interpretation provided a solid basis for a biochemical and, eventually, a chemical approach to the problem of virus receptors. Using ovomucin as substrate, Gottschalk and Lind (1949b) presented the first chemical evidence for the activity of the virus enzyme. They isolated by dialysis from the digest of ovomucin with living influenza A virus a low molecular weight compound. Concomitant with the release from the mucoprotein of the split product, and of this product only, the ovomucin irreversibly lost its hemagglutinin inhibitory capacity, whereas the virus retained its enzymatic activity. Heat-inactivated virus did not promote these changes. RDE imitated in detail the effects of living virus on ovomucin. The split product resembled *N*-acetylglucosamine with regard to reducing power, nitrogen content, and absorption spectrum of the purple-colored compound formed in the Morgan-Elson reaction. However, contrary to the behavior of *N*-acetylglucosamine, the split product decomposed with discoloration on heating with very dilute mineral acid, as 2-deoxyhexoses do. In addition, it gave the direct Ehrlich reaction (without alkali pretreatment). The same product was obtained when the electrophoretically homogeneous urine mucoprotein was acted upon by highly purified influenza B virus (Gottschalk, 1951). The weight of the product split off amounted to about 1 % of the substrate used. These results, taken together with the biological effects of RDE, indicated that the binding power of the cellular receptors for the influenza virus and the competitive hemagglutinin inhibitory capacity of the soluble receptors (mucoproteins) were closely associated with the

product released enzymatically from the inhibitory mucoproteins. Subsequent work was, therefore, focused at the identification of the split product, the elucidation of its molecular structure, and its location and linkage within the inhibitory mucoids.

The split product shared its unusual properties with an acid first isolated in crystalline form by Blix (1936) from bovine salivary mucin. This acid was shown to have the composition $C_{13}H_{21}O_{10}N$ and to contain an *N*-acetyl group, an *O*-acetyl group (which was very easily lost), a reducing group, a primary alcohol group, and a carboxyl group (Blix *et al.*, 1955, 1956). Digesting urine mucoprotein with the virus enzyme, Klenk *et al.* (1955) confirmed Gottschalk's results, crystallized the split product, and identified it as *N*-acetylneuraminic acid. Neuraminic acid was first isolated as the methoxy derivative from brain gangliosides (Klenk, 1941). The properties of *N*-acetylneuraminic acid (NANA) suggested that it differed from Blix's acid only by the lack of the *O*-acetyl group. Therefore, from Blix's data it would have the formula $C_{11}H_{19}O_9N$; but Klenk *et al.* (1955) assigned to NANA the formula $C_{12}H_{21}O_{10}N$. This discrepancy and the many possible interpretations of such formulas made it impossible at this stage to conceive a molecular structure of neuraminic acid. Obviously the preparation from acetylated neuraminic acid of a structurally well-defined degradation product was required. This information was provided by the isolation of pyrrole-2-carboxylic acid (PYCA) from alkali-treated bovine submaxillary gland mucoprotein (BSM) and urinary mucoprotein (UM) and by the degradation of the enzymatically released split product to PYCA by weak alkali (Gottschalk, 1953, 1954b, 1955a). On the basis of these data NANA was visualized as an aldol condensation product of *N*-acetylhexosamine with pyruvic acid, and neuraminic acid as the corresponding condensate of hexosamine with pyruvic acid; the degradation of NANA to PYCA, involving loss of acetyl and of water and reverse aldolization, was depicted, as shown in Fig. 1 (Gottschalk, 1955b, 1956a). The correctness of the proposed structure was strongly supported by the synthesis in 20 % yield of PYCA from D-glucosamine and pyruvic acid (Gottschalk, 1955b, 1957a) and by the fragmentation of NANA to *N*-acetylglucosamine, CO_2, and a 2-C compound (Kuhn and Brossmer, 1956b). It was conclusively proved by the chemical synthesis of NANA from *N*-acetyl-D-glucosamine and carboxylated pyruvic acid (Cornforth, *et al.*, 1957). The crystalline synthetic product was identical with crystalline NANA from biological sources with regard to melting point, elementary analyses (C, H, N), infrared spectrum, X-ray powder diagram, specific optical rotation, and chromatographic behavior (Cornforth, *et al.*, 1958). By the synthesis, the general structure of NANA and its stereochemistry at C_6, C_7 and C_8 were defined. Recently Comb and Roseman (1958) have shown that the N-acetylhexosamine fragment of NANA is N-acetylmannosamine. Apparently under the alkaline

conditions of the chemical synthesis and fragmentation of NANA epimerization had taken place. The OH- group at C_4 of NANA is in cis-position to the acetamido group (Kuhn and Brossmer, 1957). The results of periodate oxidation would indicate that NANA in aqueous solution

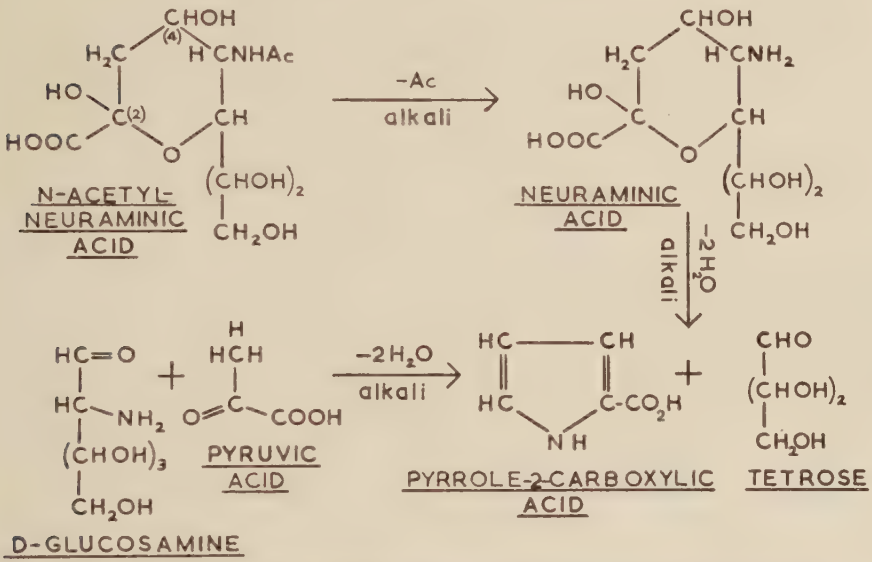

FIG. 1. Degradation of N-acetylneuraminic acid to pyrrole-2-carboxylic acid.

exists predominantly in the pyranose form and that ON-diacetylneuraminic acid has the O-acetyl group attached to C_7 (Blix *et al.*, 1956; Klenk *et al.*, 1956).

Sialic acid, the group name for ON-diacetylneuraminic acid, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, and other acylated neuraminic acids (for nomenclature see Blix, *et al.*, 1957), is an intrinsic component of all hemagglutinin inhibitory mucoproteins (Odin, 1952, 1957; Gottschalk, 1955a, 1956a; Werner and Blix, 1956). NANA has been obtained crystalline from UM, serum mucoprotein, from the inhibitory mucoproteins present in meconium, ovarian cyst fluids, human cervical mucus, human milk, and from ovomucin; diacetylneuraminic acid from BSM (Klenk *et al.*, 1955; Böhm *et al.*, 1957; Zilliken *et al.*, 1955; Odin, 1955; Blix, 1936). As product of enzymatic cleavage (virus or vibrio enzyme) NANA has been obtained with BSM, UM, and serum and meconium mucoproteins as substrates (Faillard, 1957; Böhm *et al.*, 1957; Zilliken *et al.*, 1957). Moreover, Klenk and Lempfrid (1957) were able to split off NANA from human erythrocytes with RDE and to crystallize

the compound. This contribution proved beyond doubt the previously postulated chemical analogy between cellular receptors and soluble inhibitory mucoproteins, an analogy also reflected in the reduction of the net negative surface charge of both human erythrocytes and inhibitory mucoproteins upon RDE action (Hanig, 1948; Ada and Stone, 1950; Pye, 1955). It is of interest to note that the sialic acid of all potent hemagglutinin inhibitors is NANA. Thus, lipid-free extracts of equine erythrocytes (stromata), known to contain *N*-glycolylneuraminic acid, are devoid of inhibitory power, whereas the corresponding extracts of human erythrocytes, containing NANA, inhibit virus hemagglutination (Yamakawa, 1956). This observation may have some bearing on the fact that equine red cells, in contrast to human red cells, are not readily agglutinated by influenza virus (Clark and Nagler, 1943). Another example is BSM. This mucoprotein has the highest sialic acid content known so far (17 %), of which at least 80 % is terminal (Gottschalk, 1956b). Yet, its virus hemagglutinin inhibitory power is very limited. BSM efficiently inhibits only influenza PR8 indicator virus; LEE indicator virus is inhibited very slightly, and eight other influenza virus strains are not inhibited at all. When living PR8 or LEE virus was allowed to act on BSM, no significant change in the net negative charge of the muco-protein was observed (Curtain and Pye, 1955). According to Blix (1958) the sialic acids of BSM are a mixture of ON-dilacetylneuraminic acid, N-acetyl-O-diacetylneuraminic acid and N-glycolyneuraminic acid.

With regard to the position and linkage of sialic acid in inhibitory mucoproteins, it was shown by Gottschalk (1956b) that in BSM sialic acid occupies a terminal position and is joined through the potential keto group glycosidically to the adjacent unit. RDE released 64 % of the total sialic acid present in BSM (Gottschalk, 1955b, 1956b). Heimer and Meyer (1956) and Faillard (1957) arrived at the same results. With UM as substrate, both the influenza virus enzyme and RDE split off 50–60 % of the total sialic acid present, and sialic acid only (Klenk *et al.*, 1955; Faillard, 1957).

The over-all structure of the soluble receptors was early recognized (Gottschalk, 1952) as that of conjugated proteins, containing as prosthetic groups relatively small sized polysaccharides or oligosaccharides. In the case of UM, the prosthetic group, detachable by alkali, consists of galactose, mannose, fucose, glucosamine, galactosamine, and NANA in the molar ratios 6 : 3 : 1 : 6 : 2 : 3. The molecular weight of the prosthetic group is of the order of 12,300 (assuming acetylation of the amino sugars). Since the molecular weight of UM is 7×10^6 and its carbohydrate content about 21.5 % (calculated as anhydro sugar), approximately 120 individual prosthetic groups are assumed to be attached to the protein core (Gottschalk, 1958). BSM contains 11.4 % *N*-acetylgalactosamine and 17 % *ON*-diacetylneuraminic acid, i.e., about equimolar quantities of the two components; in addition,

small amounts of galactose (0.7 %), mannose (0.2 %), fucose (0.7 %), and *N*-acetylglucosamine (1.0 %) are present (Gottschalk and Ada, 1956; Heimer and Meyer, 1956). By very gentle alkali treatment, Gottschalk (1957c) has detached a disaccharide from BSM. This reducing disaccharide was recently obtained in an analytically pure state; its formula is $C_{19}H_{32}O_{14}N_2$ and it consists of NANA linked ketosidically to C_6 of *N*-acetylgalactosamine (NAGal), as shown in Fig. 2. The viral enzyme and RDE split the disaccharide into NANA and *N*-acetylgalactosamine. (Gottschalk and Graham,

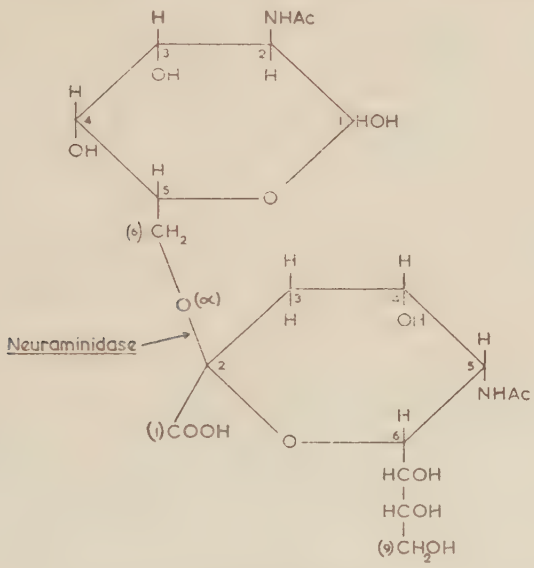

Fig. 2.

1958). By this action the enzyme is characterized as an *O*-glycosidase (ketosidase). Since both the viral enzyme and RDE invariably release from their substrates a terminal, ketosidically linked, acylated neuraminic acid, the enzyme has been termed "neuraminidase" (Gottschalk, 1957b).

From the evidence presented it would appear that the soluble influenza virus receptors (hemagglutinin inhibitors) are conjugated proteins with oligosaccharides as prosthetic groups. The size of the oligosaccharide may vary with the type of mucoprotein, and so may vary the number of prosthetic groups. Acetylated neuraminic acid residues are terminal units of the oligosaccharides; these terminal units are joined through a neuraminidase-susceptible glycosidic linkage to an adjacent sugar residue. There is every

reason to believe that the cellular receptors are built up in a similar way. Both the terminal neuraminic acid of the prosthetic group and the polypeptide core providing the framework for an orderly spatial arrangement of the prosthetic groups are essential for attracting and binding the influenza virus particle. Enzymatic removal from the prosthetic group of the terminal acetylated neuraminic acid deprives the receptor of its binding power. Breakdown of the protein framework has the same effect. Thus, it has long been known that trypsin inactivates inhibitory mucoproteins (Burnet *et al.*, 1947; Hirst, 1948; Gottschalk and Lind, 1949a). The oligosaccharide on its own does not inhibit hemagglutination by indicator virus, as was shown with the disaccheride (Fig. 2) and with sialyl-lactose. This trisaccharide, which is present in milk, consists of diacetylneuraminic acid and lactose (Kuhn and Brossmer, 1956a). In this case, neuraminic acid is linked ketosidically to C_3 of galactose, the ketosidic bond being of the α-type (Kuhn and Brossmer, 1958; Gottschalk, 1957b). The trisaccharide is susceptible to virus and *V. cholerae* neuraminidase.

Just as different mucoproteins offer different structural situations to one and the same virus, different strains of influenza virus present different surface structures to one and the same receptor. Differences in reactivity of the various influenza virus strains with one receptor, the red cell receptor, were first observed by Burnet *et al.* (1946). They found that red cells from which one strain of virus had become eluted were still agglutinable by certain other strains. Graded decrease by RDE of the number of intact receptors available at the red cell surface rendered in a definite order one virus strain after the other unable to agglutinate the increasingly impaired red cell (receptor gradient; for details see page 29). The physicochemical expression of this stepwise decrease in intact receptors available or, as we know now, of the stepwise loss of sialic acid residues is the gradual decrease in the net negative charge of the red blood cells. Thus, the electrophoretic mobility of human erythrocytes is reduced from the normal value of -1.30×10^{-6} cm.^{2}/sec.$^{-1}$/volt^{-1} to a value characteristic for each virus strain of the gradient, the lowest value of -0.37×10^{-6} cm.^{2}/sec.$^{-1}$/volt^{-1} being attained with swine influenza virus (Ada and Stone, 1950). RDE action on human erythrocytes reduces their electrophoretic mobility to -0.17×10^{-6} cm.^{2}/sec.$^{-1}$/volt^{-1}, i.e., by 87 %, indicating that the net negative surface charge of human erythrocytes is due nearly exclusively to the dissociated carboxyl group of sialic acid ($pK' = 2.60 \pm 0.05$ at 20°C. and $0.05N$). The receptor gradient probably reflects the degree of steric hindrance to the close fit between the complementary combining groups of virus and receptor, exerted by the surface structure of the individual virus strains. In a similar fashion Burnet (1955) has invoked differences in "accessibility" of receptors to account for the gradient. Since the surface structures of the virus receptors, whether

cellular or soluble receptors, vary, it might be expected and was substantiated experimentally (Stone, 1949b; Burnet, 1949) that the position of the virus strains at the gradient varies with the type of receptor taken as reference substance.

If the phenomenon of hemagglutination by viruses (see page 21) is interpreted to mean the existence of specific receptor groupings at the red cell surface for the virus concerned, then many other receptor substances must exist. This may be postulated from the fact that RDE pretreatment of the erythrocytes does not impair hemagglutination by quite a number of animal viruses outside the myxovirus group. Some indication of the chemistry of these postulated receptors is available for Theiler's encephalomyelitis mouse virus. Mandel and Racker (1953a,b) have isolated and purified from intestinal tissue of adult mice a mucopolysaccharide which inhibits hemagglutination and infectivity of Theiler's GD VII strain of encephalomyelitis virus of mice by way of virus-inhibitor combination. Color reactions indicated the presence in the mucopolysaccharide of galactose, hexosamine, methylpentose, and hexuronic acid. The biological activity of the mucopolysaccharide was destroyed by periodate oxidation, but was unaffected by RDE.

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Chapter V

The Morphological Approach

F. B. Bang

Johns Hopkins School of Hygiene and Public Health, Baltimore, Md.

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I. Introduction

The flood of new studies on cell pathology which has followed the introduction of phase microscopy of living cells, the electron microscopy of thin sections, the staining of viral antigens with fluorescent antibody, and, finally, the discovery of a variety of new viruses of man and other animals is sufficient reason for an attempt at a complete reorientation of our ideas of cell pathology. There remains a series of classic contributions which have been thoroughly reviewed in the past (Ludford, 1951; Cowdry, 1928; Rivers, 1928), and which this review will not attempt to replace. However, as information concerning the action of virus and reaction of the cell accumulates, a new framework of reference is needed. Reappraisal could hopefully stimulate further work, but at the least it may designate the many situations in which recorded observations are lacking.

We will attempt to describe and analyze the morphological events which are involved in the absorption of virus to a cell, in the action of and reaction to the virus in the periphery of the cell, in the cytoplasm and its organelles,

and within the nucleus. This will be done for seven general groups of viruses: (1) psittacosis viruses or Miyagawanella; (2) poxviruses; (3) herpesviruses; (4) myxoviruses; (5) adenoviruses; (6) polioviruses; and (7) tumor-producing viruses. The last group, which in our treatment includes the agents responsible for mammary tumor in mice, the chicken sarcoma (Rous), and the Lucké frog carcinoma, is the least coherent, but, despite the greater coherence of the others, a number of differences within each group will be readily apparent. For the sake of completeness, some recent work on other viruses will be included. There will, however, be no attempt to review the older literature concerning encephalitis, rabies, and foot-and-mouth disease.

II. Normal Cell

Newer concepts of the anatomy of the cell (Porter, 1956; Sjöstrand, 1956), as seen with the approximately hundredfold increase in resolution available with the electron microscope, need brief review here (Fig. 1). We will attempt

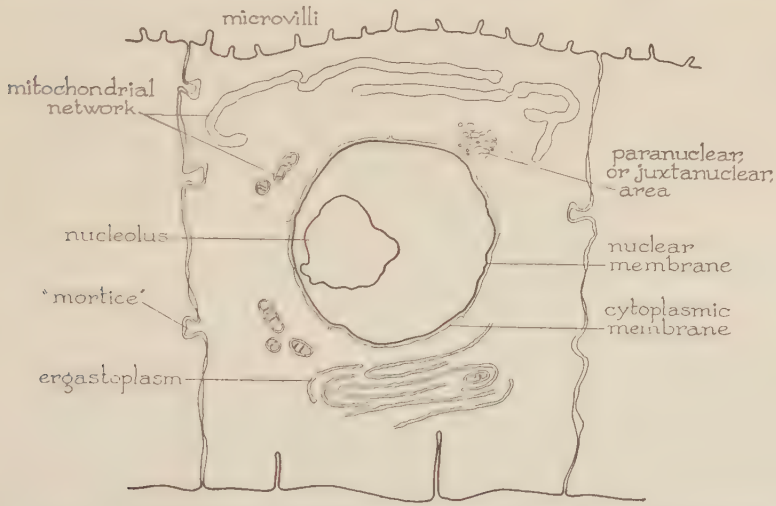


Fig. 1. Diagram of generalized epithelial cell showing variety of organized structures. Mitochondrial network indicated at upper surface is in other cells broken up into more discrete units. Continuity of ergastoplasm with cytoplasmic portion of nuclear membrane although not indicated here probably present in many cells. For purposes of reproduction double mitochondrial membranes are separated by greater distance than actually present.

to relate them to the much more dynamic picture, as seen in the living cell (Fig. 2) (Lewis and Lewis, 1924; Gey, 1956) but with less resolution. We hope to avoid the controversy concerning the "Golgi apparatus" (Pollister and Pollister, 1957) by referring to the paranuclear area simply as such, and in

this review we intend merely to describe the changes which have been found there.

In the accompanying diagrams we have limited the description to a cell in the intermitotic phase of its life. We have excluded any specialized functions. Yet, in the case of the tumor cell, major emphasis has rested with the abnormal behavior of the nucleus during division, and it is primarily on the differentiated or specialized cell that a virus acts in the intact animal. Despite the recent advances, there remains little new in our knowledge of the morphological effects of viruses on specialized cells.

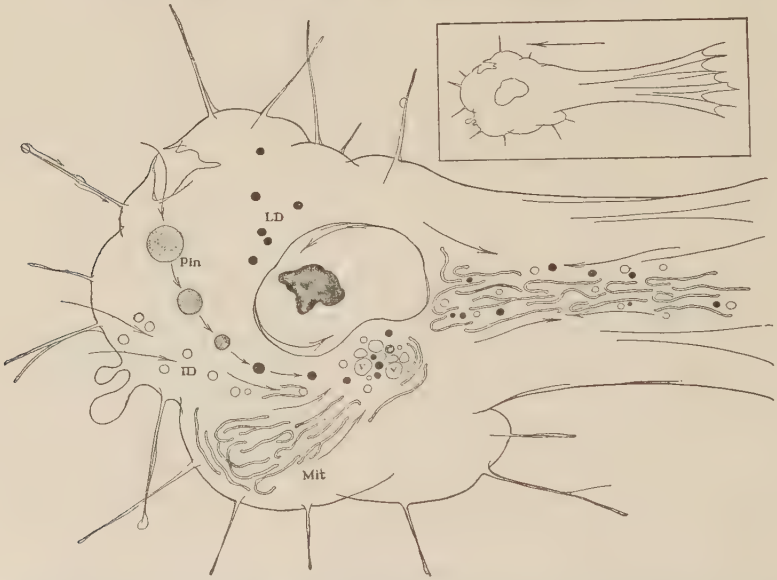


Fig. 2. Diagram of tissue culture cell showing varieties of cell activity. Process of pincytosis (Pin) indicated as bringing vacuoles into paracentral area of cell where they associate with lipid droplets (LD) and other inclusive droplets (ID). Continual extrusion of microfibrils from surface of cell, the accumulation of fluid which is milked toward the cell is indicated at left. Rotation of nuclei within cell indicated by arrows.

Some morphological aspects of cell physiology also need brief review before proceeding. The change in shape which occurs when compact, rounded cells are placed on a new surface, such as glass, has been studied particularly in the blood cells. This change is often accompanied by the loss of granules, the formation of long cellular projections, and, of course, an extreme flattening of the cytoplasm. Cells may, however, be equally extended in the intact animal when covering a surface such as the alveolar cells of the lung (Karrer, 1956) or the endothelial cells of blood vessels (Palade, 1956). Thus, tissue culture cells do not necessarily differ from similar cells in shape and relation to other cells.

A. Pinocytosis

Pinocytosis (cell-drinking) was first described by Lewis (1931) in rat macrophages in tissue culture in autologous sera. It has since been found in many different types of cell cultures, and has even been studied in cells within the animal (Algire, 1957). Small droplets, apparently projecting into the cell, have been found in electron microscope pictures (Palade, 1956), and these may represent the same phenomenon at a smaller size level. It has been proposed that this process may be an important mechanism of virus entry into the cell (Bang and Gey, 1951). The relationship of pinocytosis to phagocytosis, which may also occur in a number of cells (Fell and Brieger, 1947; Lewis and Lewis, 1924) besides the macrophage, is not known. Pinocytosis may be greatly increased in tissue culture with various types of sera (Rose, 1955), or with sera which had been injected into the host from which the cells were taken (González-Ramírez *et al.*, 1956).

B. Mitochondria

Although mitochondria may be altered by substances which damage the cell and have been found to vary tremendously with different physiological activities of cells, little is known of specific physiological effects. The capacity of coenzyme A to block the fragmenting effect of 6-mercaptopurine on mitochondria and to cause the mitochondria to become longer and thicker may be one such effect (Biesele, 1955).

C. Nucleus

The appearance of a normal nucleus has been subject to much discussion. The picture presented here is now generally accepted. The nucleolus, however, has in tissue culture been shown to vary considerably. A grossly rather coiled appearance which was seen clearly in early, relatively thick sections studied in the electron microscope (Borysko and Bang, 1951; Bernhard *et al.*, 1952), has been shown at higher resolution to consist of many fine dots or cross sections of threads grouped together in varying concentration. Of particular interest, however, are the studies on the appearance of the nucleolus in tissue culture when the cultures are treated with various purines (Hughes, 1952a; Lettré and Siebs, 1954). With the addition of adenosine, the nucleolus, which in actively growing cultures is normally homogeneous, changes to a fragmented and frequently coiled structure. This change may be related to nucleolar changes which have been described in infections with the virus of fowlpox (Bang *et al.*, 1951) and rabbit fibroma (Bernhard *et al.*, 1955).

D. Secretion

The processes of cell secretion are of obvious importance for virus release. For example, the fact that the mammary cell surface is constantly broken

down during the secretion of milk offers a mechanism of virus release which might occur in infection with the mammary tumor agent so that no pathological changes in the cell would be necessary for even a massive release of the virus. The continuous formation and excretion of fairly large droplets of mucus again offers ready exit for virus. Actual visualization of such processes may be difficult. The collection of a variety of special secretory droplets in the cytoplasm offers a particular challenge to anyone trying to identify viruses. Many of these occur in clumps (Bargmann and Knoop, 1957) and may even have dense centers within the individual particle (DeRobertis and das Ferreira, 1957). There is no single method whereby such particles may be differentiated from viruses.

The extensive knowledge of the sequence of changes occurring in mitosis has been of little use in cell pathology in relation to viruses because of almost complete lack of knowledge of changes produced by virus infections. This, in turn, is due to the fact that most of the virus infections studied have killed the cell before mitosis took place. Probably virus tumor cells would show interesting changes.

E. Osmotic Changes

The demonstration with the electron microscope of a detailed lamellar system within all kinds of cells raises interesting questions concerning fluid localization. A recent study of kidney function (Rollhauser and Vogell, 1957), in which the tubular cells during secretion show great accumulation of fluid within these spaces, has particular interest in relation to the pathological accumulation of fluid (see Section III).

III. Pathological Aspects of Cell Morphology

General knowledge of the morphological changes occurring in sick cells is an assumed prerequisite for the interpretation of the changes brought about by viruses. Furthermore, one expects some structural change, however small, to accompany biochemical change. Yet, a large proportion of the studies in nonviral cell pathology have come about as by-products of tissue culture observations (Fischer, 1946), or as controls of one sort or another for virus infections (Blackman, 1936). There are a number of studies on the effects of various mechanical traumas on larger cells (Cameron, 1952), but since most of these deal with marine invertebrate or plant cells, the direct applicability of them to our problem is not clear. For these reasons, we are unable to summarize in any logical fashion the progression of changes which occur between early derangement and final death, but we will attempt to classify the types of change found and discuss them briefly.

A. Cell Surfaces

Although probably one of the commonest and earliest reactions to injury is the formation of multiple thin extrusions of various lengths and thicknesses, this formation has not been purposefully studied in detail. These extrusions may simply represent an immediate hyperplasia of the intercellular processes or extensions of the normal microvilli which occur commonly on epithelium. Extensive bubbling of the cell surface is seen in the last stages of "pyknosis" (Bessis, 1956), but is also a common accompaniment of the last stages of mitosis (Hughes, 1952b) except in hemopoietic cells (Rondanelli *et al.*, 1956). It is likely that the numerous bits of ballooned cytoplasm seen so typically in the electron microscope (Murphy and Bang, 1952) rise from cells degenerating in this manner.

B. Osmotic Imbalance

The accumulation of fluid droplets within the cell under both physiological and pathological conditions has been observed frequently (Lewis and Lewis, 1924). In a tissue culture study of the effect of hypotonic and hypertonic solutions on chick fibroblasts, Hogue (1919) described and illustrated the changes with drawings. In hypotonic solutions, the colonies increased in size, but the cells accumulated fluid in cytoplasmic vacuoles and died early. A large vacuole situated next to the nucleus was frequently found. The neutral red vacuoles and granules lost their color. The mitochondria were not affected at first, but as the cell died vesicles formed at the extremities and persisted after the mitochondria disappeared. It is likely that the newer understanding of the detailed series of lamellae and minute intracellular channels within the cell will allow an explanation of the accumulation of fluid in between these "spaces" within the cell when osmotic imbalance occurs. This would be the simplest explanation for the distended "endoplasmic reticulum" seen in a destructive cell lesion such as influenza in the ferret nasal epithelium. Fluid under these conditions may remain accumulated in large vesicles, forcing a separation of the nuclear and cytoplasmic portions of the so-called double nuclear membrane (Hotz and Bang, 1957).

Hogue (1919) further found that cells grown in hypertonic media usually contracted; the thin processes became long and threadlike and were later drawn in. Neutral red staining showed that channels formed in the cell, and the cytoplasm became alveolar in the terminal stages. Little droplets are shown to form at the cell edge and often attach to the cell by fibrils. Again, the mitochondria were not affected significantly.

It would be of great interest to measure the density of fluid within these droplets by Barer's recent interference microscope methods (Barer and Joseph, 1955), particularly since it is possible to follow the change in density

of pinocytotic droplets as they are carried to the center of the cells (Gey *et al.*, 1955).

C. Mitochondria

The mitochondria have long been recognized as sensitive indicators of cell metabolism, but this phrase has had little specific meaning. The recent knowledge of their detailed structure and of the localization of numerous enzymes in them would in itself raise questions concerning the nonspecific implications of the quotation. Furthermore, it is now clear from several studies that extensive changes may occur in a virus-infected cell without any apparent change in the mitochondria. Thus, the future aim will be to describe specifically what changes, if any, are caused by viral lesions. Ballooning of mitochondria occurs frequently in degenerating cells. That this osmotic lesion occurring in specific organelles may not be a distinctively degenerative change is indicated by the fact that it may be induced by thyroxine (Schulz *et al.*, 1956), and that mitochondria may be specifically kept from swelling due to thyroxine by the production of anaerobiasis (Lehninger and Ray, 1957). Furthermore, Luft and Hechter (1957) report that the distended vacuolar mitochondria seen in electron micrographs of adrenal glands obtained from slaughter houses may be converted to a normal appearance by the perfusion with warm, oxygenated beef blood. Such changes are then significant in relation to the idea that tumors may involve lesions of the mitochondria. Degeneration or disappearance of the mitochondria in dying cells has also been described (Lewis, 1923; Lewis and Lewis, 1924).

D. Paranuclear Hypertrophy

Since the discussion concerning the nature of the "Golgi apparatus" continues (Pollister and Pollister, 1957; Baker, 1957), we will limit this review to a discussion of the tissue culture findings concerning the paranuclear area. It is particularly in the tissue cultures of poliomyelitis infections that lesions have been described. Lewis (1920) described "giant centrospheres in degenerating mesenchyme cells." The term "centrosphere" was used to designate a peculiarly differentiated region about the centriole. The lesions range from slight vacuolization around the centrosphere to an enlarged centrosphere with sharp borders, medullary and cortical zones, and concentric or radial arrangement of mitochondria about the centrosphere. Lewis emphasized the importance of this degenerative lesion because of an apparently identical phenomenon in certain cancer cells.

E. Hypertrophied or Giant Cells

The presence of giant cells in tissue cultures of all kinds is not rare (Lewis and Lewis, 1924). It was early described by Lambert and Hanes (1911) in

cultures of carcinoma and sarcoma and later in spleen, especially in response to such foreign bodies as lycopodium spores and cotton fibers (Lambert, 1912). Giant cells may form with the stimulus of tubercle bacilli (Timofejewsky and Benewolensjkaja, 1925), as the result of X-ray (Puck and Marcus, 1956; Pomerat *et al.*, 1957). Abnormal mitosis is produced by sublethal amounts of mustard gas (Fell and Alsopp, 1948). The basic reason for this imbalance between nuclear and cytoplasmic division is not known. Giant cells may arise either by a merging of cells or by failure of cytoplasmic division.

F. Amino Acid Deficiencies

Demonstration of the amino acid requirement of cells (Kieler, 1953, 1954; Eagle, 1955), and that there are certain vitamin requirements (Eagle, 1955a), has been accompanied by the suggestion that the different amino acid deficiencies produce morphologically different cellular lesions. In such examples as arginine deficiency, cell degeneration was so pronounced that only sudanophilic vacuoles and nuclei remained (Kieler, 1953). However, high resolution phase microscopic studies have not been reported.

G. Cytoplasmic and Nuclear "Degenerative" Changes

In an excellent and somewhat neglected study of the changes which occur when tissue cultured cells are not refed, Horning and Richardson (1929) followed their morphology, both in the living state, with Janus green and neutral red dye, and in the fixed state, after osmic acid vapor fixation and staining. Chick embryo heart cells, kept for 50 hours and more in unchanged media, became elongated and developed needle-like processes which often had swollen tips. Pieces of these extrusions broke off. There was extensive pseudopodial activity, with bizarre branching and sporadic division of nucleoli. By the third to fourth day, masses of lipoid had accumulated, the cell contour had changed, but "it is interesting to observe that up to this advanced stage of cellular degeneration that occurs after 7–8 days *in vitro*, the nucleus remains relatively unaltered. The nuclear membrane is intact until the cytoplasm has lost all of its distinguishing features." Chromatolysis took place at 7–9 days, but the nucleolus persisted. In the final stages of cytolysis, mitochondria and neutral red granules still persisted. The degenerative changes in undifferentiated cells were essentially similar but differed somewhat in sequence. In a cytochemical study of pycnotic nuclear degeneration, Leuchtenberger (1950) followed the changes with a photometric Feulgen method for DNA. She found that the nuclei of sarcoma 180 and liver cells, when killed and transferred subcutaneously, progressively shrank, the chromatin became homogenous, and the nucleoli disappeared. The protein was initially reduced to one-half; later, there was a loss of DNA.

Intranuclear inclusions have been induced in cells with a variety of chemicals (Blackman, 1936). Deoxyribonucleic acid was not found in these or in cytoplasmic inclusions induced in the same way (Wolman, 1954) (1955), but, as will be pointed out later, it is also absent from the late lesions of a typical "intranuclear virus" such as herpes.

IV. Bacterial and Rickettsial Infections

Although detailed studies of the pathology of cultured cells are not extensive in bacterial or rickettsial infections, a brief review is instructive. Bacteria may be ingested and may accumulate in great numbers in cells without destroying the host cell (Fell and Brieger, 1947), or they may be digested fairly rapidly if nonpathogenic (M. R. Lewis, 1923), or they may rapidly destroy the host cell (M. R. Lewis, 1920).

The behaviour of rickettsia in living rat fibroblasts has even closer analogies with the effects of viruses. *Rickettsia rickettsii* remains scattered through the cytoplasm and causes fairly rapid degeneration of the host cell; it also penetrates into the nucleus of the host cell. *R. tsutsugamushi*, on the other hand, tends to accumulate in a mass near the nucleus and does not cause gross morphological changes in the host cell for 8 or 9 days. The rickettsiae are fairly frequently found extruded in the microfibrils, which are often formed by these cells (Schaecter *et al.*, 1957).

R. burneti was studied in chick heart and skeletal fibroblast cultures (Kausche, 1952). In these host cells, large and small clear vacuoles were formed and within them were long chain forms of rickettsia. The nucleus was pushed to one side by the enlarging vacuoles. When Aureomycin was added, the vacuoles disappeared. Photomicrographs taken at 257 mμ (ultraviolet) showed very dark nucleoli, presumably indicating an increase in nucleoprotein metabolism. Electron microscope studies of thin sections of *R. mooseri* (Wissig *et al.*, 1956) in yolk sac showed the rickettsiae in mesothelial and epithelial cells, but not in fibroblasts, macrophages, or vascular cells. The rickettsiae merely seem to grow and replace the host cell with little reaction, leaving a pycnotic nucleus and a few mitochondria which showed no lesions even when they had been greatly reduced in number.

V. Psittacosis Group Viruses (Miyagawanella)

A. Tissue Culture Stages

The development of the viruses of this group in cells was clearly described before the advent of the electron microscope. Furthermore, Bland and Canti (1935) had been able to follow part of the developmental sequence in individual living cells. This was combined with a study in tissue culture of chick

lung epithelium, removed at frequent intervals after infection with large amounts of psittacosis virus and carefully stained. Since most, if not all, of the cells were infected, certain fairly definite conclusions could be made concerning a cycle of development. Although this study was an extension of the original observations of Bedson and Bland (1932), we will use the later outline:

1. During the first 8 hours an indeterminate stage occurs, during which good elementary bodies are found on the outside of the cells, but no recognizable changes inside.

2. From 8 to 24 hours, a stage of homogeneous plaques is observed. These are scanty and small and at first are found mainly at the tips of the cell process. Later they become larger and more central. It is important to note that these plaques are only apparently homogeneous, for after strong decolorization they are seen to consist of a pale pinkish matrix in which there are lilac-colored elementary bodies. Thus, the plaques are considered as colonies of the large forms of the virus.

3. Between 18 and 24 hours, colonies of large forms are prevalent.

4. From 24 to 48 hours, the picture becomes more varied. The colonies contain particles of intermediate size and depth of color.

5. From 48 hours on, the elementary bodies become more and more numerous. The particles become more motile (Brownian movement), presumably because of a change in the viscosity of the matrix.

Despite the valuable data which may be obtained from the study of living infected cells, particularly when the virus is large enough to be seen with a light microscope, there have been relatively few such studies of this virus group. Gey and Bang (1939) cultured the virus of lymphopathia venerea (lymphogranuloma) in a strain of a human fibroblast and found that clear vesicles developed in cells some 12 days after inoculation. Enlarged virus particles of a little less than 1 μ developed within the clear fluid, and in turn developed into masses of smaller particles of about 0.2 μ, which pushed the nucleus to one side, hypertrophied into masses several times the size of the normal cell, and yet usually maintained an active Brownian movement. Virus release took place with an obvious breakdown of the vesicle. The process of infection was followed in the same cultures for more than 7 months. No plaques were described. Similar changes were later found in malignant rat cell strains infected with the same virus (Bang and Gey, unpublished). Almost identical findings were reported independently by Manabe (1939).

B. Elementary Bodies

Since the first descriptions of the tiny granules associated with this group of viruses (Levinthal, 1935; Miyagawa *et al.*, 1936), there has been a series of

such descriptions. Most of these, however, are of little value in cell pathology, for they are usually based on smear preparations. Miyagawa *et al.* (1936) described the occurrence of granulocorpuscles in tissue culture cells. The complete cycle was followed by Rake and Jones (1942) in infected yolk sac cells. During the first 6 hours, occasional virus bodies or elementary bodies could be seen in smears or sections, and were thought to represent the original massive inoculum. This apparent virus then disappeared, and initial bodies of about 1 μ in diameter were found in the cell. These seemed to divide like cocci and to form pairs. Later they appeared in vesicles surrounded by a thin matrix. After 20 hours of infection, bright red elementary bodies were found within large green plaques (Noble's stain). Several plaques might occur within one cell. Some individual plaques attained a diameter of 4 to 7 μ.

There have been several studies with the electron microscope, but in general these have not been adequately correlated with simultaneous observations on living or light microscopic stained material. Gaylord (1954) studied the appearance of the intracellular virus in cells of the chicken chorioallantoic membrane at 48, 72, and 96 hours after heavy inocula. Since the minimal time here exceeded by far the stages of infection described by Bland and Canti (1935), no true sequence of intracellular changes could be determined. However, the increased resolution afforded by electron microscopy in this and in subsequent studies brought out a number of points that were not clear previously. The inclusions contain a collection of particles varying from large, rather homogeneous ones (which seem to be 250 to 500 mμ in cross section and would then correspond to the large form of the virus) to denser, smaller particles with dark centers which are scattered among them. These latter are identical in appearance with the virus elementary body which has been carefully identified by Crocker (1954) by a series of correlative studies of virus suspensions in the electron microscope. Undoubtedly, they are the smaller elementary bodies. Intermediate forms and so-called incomplete forms are also described (Tajima *et al.*, 1957). Secondly, the virus may appear free in the cytoplasm or be contained in a vacuole in the cytoplasm of the host cell, as has been seen in living cells. The wall of the vacuole has no characteristic different from the cytoplasmic membrane. The host cytoplasm, mitochondria, and nucleus are free of changes. Although the latest study (Tajima *et al.*, 1957) does include samples taken at frequent intervals after infection with large amounts of virus, no certain *sequence* of changes of the virus elementary bodies was demonstrated.

It seems clear from the sum of these studies that two forms of virus particles may be identified. First is the larger form (Bedson and Gostling, 1954; Bland and Canti, 1935; Swain, 1955) and, second, the smaller characteristic elementary bodies. The relationship of the latter to the plaques is not

always clear. There is good evidence that the plaques contain the elementary bodies within them (Bland and Canti, 1935; Rake and Jones, 1942; Weiss, 1949), but the origin of plaques is obscure. They are not always present even in stained material (Burnet and Rowntree, 1935), and whether they contain material excreted from the virus or products of cell reaction is not known. For this reason the studies on the appearance of the virus during various types of chemotherapy are particularly valuable. Weiss (1950) determined the effect of penicillin and Aureomycin (Gogolak and Weiss, 1950) on embryo-adapted feline and murine pneumonitis; in the embryonic cells they observed that when the antibiotic "prevented the division of the virus particle" a conglomerate of irregularly shaped plaques developed nevertheless. Vesicles and elementary bodies developed apparently normally when the penicillin was eliminated or destroyed.

The exact mode of multiplication of these viruses in the cell is not yet clear. The claim that they may multiply extracellularly (Weiss, 1949) does not seem to be substantiated by data clearly free of artifacts. The extracellular *appearance* might also be obtained because of the extreme thinness of the alveolar walls (Karrer, 1956). A noninfectious phase (Girardi *et al.*, 1952) of the virus has been disputed (Bedson and Gostling, 1954). Electron microscope studies of the early phases of virus multiplication have been negative and have, therefore, supplied no useful data on this point. Light microscopic studies have been equally unproductive.

C. Fluorescent Antibody Staining

In a study of the development of psittacosis virus in tissue cultures of embryonic mouse liver, Buckley *et al.* (1955) found that virus antigen was detectable as soon as one hour after the inoculation of the culture with large amounts of virus. Focal accumulations of virus in the cytoplasm were common under these conditions whereas, following inocula with small amounts of virus, neither inclusions nor fluorescent antigens were detected.

VI. Poxvirus

A. Vaccinia

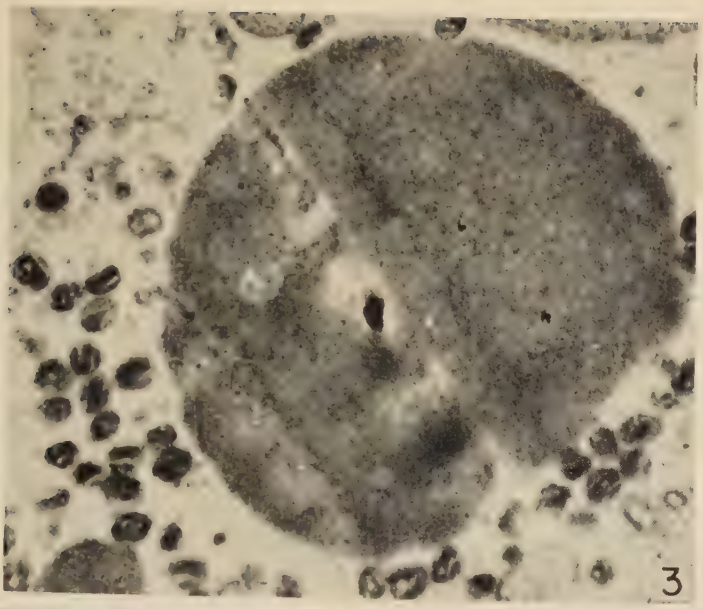
Of the animal viruses, the poxviruses (Fenner and Burnet, 1957), with vaccinia as the type species, are known in greatest morphological detail. Vaccinia is one which has also been most completely identified. Since the members of the group share a similar morphology, the process of infection with vaccinia may be followed as an example. The cytoplasmic accretions of differentially staining material, known since Guarnieri's original description (1892), have been studied with increasing detail. By 1939 (Downie, 1939;

Amies, 1938), it was generally accepted that they were composed of varying proportions of homogeneous material in the cytoplasm and that within the consequent "inclusion," numerous "elementary bodies" might sometimes be identified by appropriate staining. The studies of Bland and Robinow (1939) on rabbit corneal cells infected *in vitro* with vaccinia clearly showed that the virus particles could be recognized as they developed into colonies within the cell. These authors inoculated small explants of corneal epithelium with purified and concentrated virus for 5 minutes, then washed away the free virus. The cultures were removed at intervals, fixed with osmic acid vapor, and stained with Giemsa.

1. *Identification*

In the first stage of infection, small homogeneous bodies were found which varied in dimension from the size of an elementary body to that of a staphylococcus. As many as 67 of these were found in one cell. The larger and more distinctly homogeneous bodies appeared later. Small and medium-sized networks then developed within the inclusions and became progressively larger and more numerous during 24 hours after infection. Quantitative data on the different types and numbers of inclusions prevailing at different periods of infection were presented. The elementary bodies, which were readily distinguishable at the start of the infection, disappeared during the middle portion of the cycle and reappeared later, exclusively within the large networks. Feulgen-staining characteristics were not consistent. The elementary bodies were found Feulgen-negative, the inclusions Feulgen-positive, and the larger networks Feulgen-negative, with deep purple rods and granules within. The presence of Feulgen-positive material as part of the matrix of the inclusion was also found by Matumato and Dohi (1956) in ectromelia. In some cells infected with vaccinia the elementary bodies appeared to be released individually from the ends of long stalks (Bland and Robinow, 1939).

Eisenberg-Merling (1940) studied the same cell system but followed the development of these events in the living cells obtained by resecting bits of cornea from an infected rabbit. The cells were studied under a dark field in Tyrode's solution or saline. Changes were detectable as early as 24 hours after inoculation. The whole surface of a cell was sometimes studded with elementary bodies, which were immobile or adherent to the gel of the cytoplasm. At a later stage these seemed to be oscillating aggregations of elementary bodies, which were also found in rapid Brownian movement in different parts of the cytoplasm. Some cells had several inclusions; the smallest either contained single elementary bodies, or they were filled with dancing elementary bodies. Phase microscope studies of ectromelia in sarcoma cells have shown similar events before cell destruction sets in (Ozaki *et al.*, 1956).



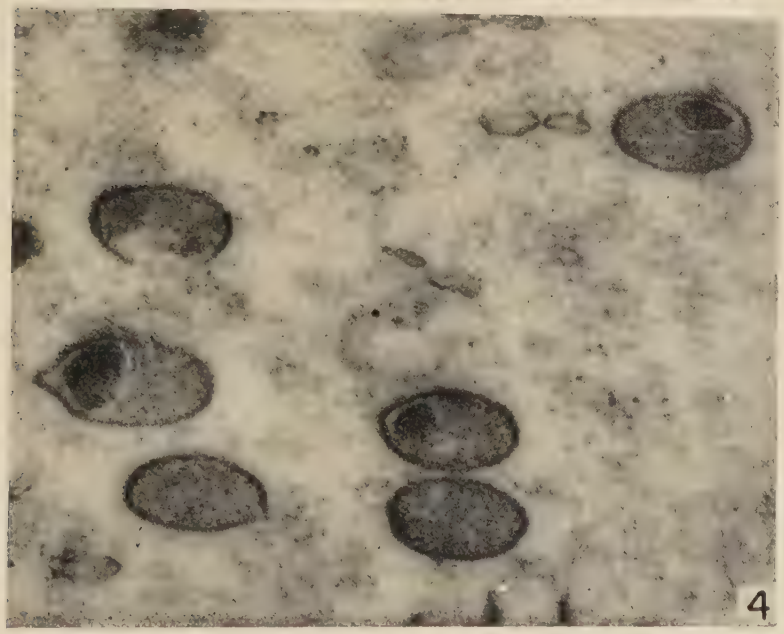


FIG. 3. Ectromelia inclusion surrounded by particles resembling mature virus. The interiors, instead of being hollow, show bars, dumbbells, and double circles (Courtesy Drs. Gaylord and Melnick, 1953) (Magnification: × 16,000).

FIG. 4. Intracytoplasmic vaccinia virus composed of characteristic granular material with an eccentric nucleus-like body (Courtesy Dr. Councilman Morgan, 1954) (Magnification: × 74,000).

2. *Electron Microscopy*

The application of the electron microscope to a study of thin sections of these infections (Bang, 1950; Morgan and Wyckoff, 1950) rapidly led to a whole new series of morphological findings (Fig. 3). The elementary bodies, which had been shown to have a dense central portion (Green *et al.*, 1942; Dawson and McFarlane, 1948), and which have been extensively analyzed by enzymatic digestion methods (Peters and Stoeckenius, 1954), were readily, identified in the tissues (Bang, 1950; Gaylord and Melnick, 1953; Wyckoff, 1951). Their fine structure has been analyzed in detail (Fig. 4) by Morgan and co-workers (1954b). In several studies (Gaylord and Melnick, 1953; Dohi, 1956; Matumoto and Dohi, 1956), large homogeneous inclusions with sharp edges have been found in the cytoplasm of the cells. In some cases, e.g., vaccinia in cornea (Gaylord and Melnick, 1953) and ectromelia in ascites cells (Dohi, 1956), the evidence seems clear that these inclusions lack any discrete particles within them; in others, there is clearly a mixture of formed elementary bodies or viruses and a homogeneous material. Finally, it is clear that, as in the epithelial cells studied by Bland and Robinow (1939), the virus may also be diffusely distributed throughout the cytoplasm. The elementary bodies of ectromelia have been found to be nicely delineated in phase microscopy of Ehrlich ascites cells infected with this virus (Matumoto and Dohi, 1956). The typical large cytoplasmic masses, seen particularly in infections with ectromelia, are probably identical with the intensively eosinophilic masses seen with the light microscope in cowpox (Downie, 1939) and ectromelia (Downie and McGaughey, 1935). However, in such a spot as the chorioallantoic membrane these must be clearly differentiated in the electron microscope from red cells which may have been manufactured *in situ* or may have been phagocytized, as in ascites tumor cells. The nature of these homogeneous bodies, which in most cases clearly are not red cells, would be of considerable interest.

Secretion of these masses, perhaps as waste material, by the cell in reaction to the altered metabolism induced by the virus is the most likely explanation of their existence. Such a possibility is supported by Matumoto's (Matumoto and Dohi, 1956) claim that two strains of ectromelia when growing within ascites tumor cells differed from each other in their relation to these "blue staining inclusion bodies" which in the one strain formed independently of the virus, and in the other were formed with a number of viral particles enclosed within.

The continuing electron microscope and enzymatic analyses by Peters (1957) of separated virus particles emphasizes that the central portion of the virus particle is not uniform. He describes it as ring-shaped. The varying fine morphology (Gaylord and Melnick, 1953; Morgan *et al.*, 1954b; Eaves and Flewett, 1954), as seen in thin sections has not yet been clearly related

to this. Morgan describes intracytoplasmic viral particles which contain dense nucleus-like bodies, which are separated from the granular ground material by a zone of lesser density. They were enclosed by a single membrane. Near the surface of the host cell they have a double membrane. Gaylord and Melnick (1953) clearly show particles with varying internal density which sometimes look almost like bars. Although a number of hypothetical sequence or life cycles of development for the individual virus units have been proposed it would seem premature to review any of these before electron microscope studies of the changes occurring within one cell cycle of multiplication are available. Furthermore, the increasing recognition of the artifacts of preparation, particularly of polymerization of the embedding material (Borysko, 1956; Watson, 1957) cautions against the danger of finding in nonpathological appearances too easy confirmation of ideas dependent primarily on studies of the infectious activity of the virus.

3. *Fluorescent Antibody Staining*

The progress of infection with vaccinia virus has been followed in cultured cells by determining the increase of antigen by Coons's fluorescent antibody technique (Noyes and Watson, 1955). This virus destroyed cells of a human epidermoid carcinoma in 48 hours. Early in infection (9 hours) a small amount of antigenic material accumulated near the nucleus. At 16–24 hours increasing amounts in the same area were sometimes sharply delimited. Nuclear staining was observed frequently in cells containing large amounts of antigen. This observation, however, must be interpreted with caution, for tumor cells frequently show nuclei notched with deep indentations of cytoplasm, and viral antigen in this cytoplasm would appear by light microscopy to be within the nucleus.

Noyes and Watson (1955) suggest, because of the presence of antigen in projections of cells, that virus transmission may take place by such cytoplasmic connections. This agrees with the several findings of particles at the tips of microfibrils (Robinow, 1950) or microvilli (Bang, 1950).

It would be profitable if the morphological data here reviewed could be correlated with virological (infectious) measurements. In a recent study, Overman and Tamm (1957) found that vaccinia virus in the chorioallantoic membrane began to increase at 10 hours; that from that time until the forty-eighth hour it increased logarithmically, and that it then continued to be produced until the third day. Nearly all of it was bound to the cells and continued to be released from cells for as long as 7 days. Clearly, then, the time period for infection of individual cells can currently be determined no more precisely than it was morphologically by Bland and Robinow (1939), who found a steady increase in inclusions and elementary bodies up to 24 hours.

B. Fowlpox

1. *Inclusion*

The fowlpox inclusion is considered separately for the sake of convenience. The lesions produced are not fundamentally different from the other members of the poxvirus group. They have been likened to the cellular lesions of molluscum contagiosum. The early studies of fowlpox have been fully reviewed by Goodpasture (1928). Fundamental knowledge concerning the interactions between virus and cell, which ultimately develop the massive lipoidal inclusion incorporating numbers of virus particles, has advanced little since Ludford's review (1951). The inclusions have not been found in fibroblasts or macrophages in tissue culture, or even in chick epithelial cells in tissue culture. This may be due to the destructive property of the virus for these latter cells when cultured (Bang *et al.*, 1951). Fibroblasts, which normally resist the virus, may be destroyed under special conditions (Kohler and Schreibel, 1956).

2. *Effect on Nucleolus*

The normally homogeneous nucleolus of these chorioallantoic cells appears in the late stages of infection of the chorioallantoic membrane as a structure of coiled or tangled strands (Bang *et al.*, 1951). Although this conversion has also been illustrated by Morgan and Wyckoff (1950), it has not been further studied. It should be reconstructed from serial sections (Bang and Bang, 1957) and compared with similar preparations of the known changes in nucleolar structure produced by adenosine and other nucleosides, nucleotides, and benzimidazole (Hughes, 1952a; Lettré and Siebs, 1954). It is possible that an excess of nucleosides is formed during the early phases of virus growth (this is indicated by the Feulgen-positive matrix of the other poxvirus inclusion), and that this influences nucleolar morphology. A similar change in the shape of the nucleolus has been reported in rabbit fibroma (Bernhard *et al.*, 1955).

3. *Development Sequence*

It is the aim of morphological studies to describe how the various units are formed. It seems to this reviewer that a premature attempt to deduce a *sequence* of changes from a *variety* of changes, when the deduction is not based even on a statistical correlation with time, is to obstruct the game. Present knowledge either of the integrity of any phase, or of the mechanisms by which the changes develop, supports no sequential conclusions. When large numbers of cells have been infected with identifiable particles and these in turn have been followed in the electron microscope during the first "cycle" of virus development, then a proposed developmental series may be

studied. One of the difficulties of interpretation is brought out by Morgan *et al.* (1955), who showed that by serial sections of vaccinia-infected cells "all particles sectioned five or six times can be shown to contain a nucleoid (nucleus) at one stage of development."

C. Shope Fibroma

Since this virus has been shown to be related to myxoma and the latter is now included with the poxviruses, some discussion of recent work on this rabbit tumor is appropriate here. Bernhard *et al.* (1955) have examined by electron microscopy some 13 fibromas and fibrosarcomas provoked by this virus and believe that the virus forms in a homogeneous paranuclear mass within the cytoplasm which they call "viroplasm." The detailed structure of the virus is like the other poxviruses. Febrve *et al.* (1957) followed the development of these masses in cell lines originating from rabbit testicles and showed that the diffuse inclusion without identifiable virus units was present as early as 5 hours after infection. This corresponded to the appearance of soluble complement-fixing antigen. Fully developed virus in large amounts was found later. The absence of virus in these inclusions is similar to the ectromelia pathology.

VII. Herpesvirus

Although the striking nuclear lesion produced by this virus was early recognized (Cowdry, 1928) and, indeed, has been used to trace the pathogenesis of the disease (Goodpasture and Teague, 1923), our information concerning the intracellular pathogenesis of the viral infection is still incomplete. There are no published studies on the microscopic effect of herpesvirus on living cells. A few strains of virus have been studied in the electron microscope on a few cell types (Morgan *et al.*, 1954a; Wyckoff *et al.*, 1956). A recent fluorescent antibody study has emphasized the peripheral presence of antigen (O'Dea and Dineen, 1957), although an earlier study has shown virus antigen in the nucleus (Lebrun, 1956).

A. Intranuclear Inclusion

The usual description of this lesion includes a separation of the eosinophilic mass from the nuclear membrane and a margination of the chromatin along the membrane. This picture probably represents the last stage in the morphological and chemical breakdown of the integrity of the nucleus (Scott *et al.*, 1953). The somewhat modified picture of the earlier lesion which may be seen in the more adequately fixed chorioallantoic membrane infections (Anderson, 1940; Crouse *et al.*, 1950), may more closely reflect the cellular changes which occur before the cell starts to disintegrate.

B. Histochemistry

Histochemical studies, including Feulgen stains, have shown a positive reaction in the nuclei of infected cells (Crouse *et al.*, 1950; Wolman and Behar, 1952) and emphasize the need to study fairly early lesions 10 to 24 hours after the infection of the chorioallantoic membrane. It is not easy, however, to determine the age of such lesions within individual cells, for tissue culture studies indicate that virus is liberated from individual cells from 6 to 12 hours after infection. Therefore, a typical pock results from several "cycles" of virus liberation. A clear correlation of the development of infectivity with the formation of a distorted nuclear pattern preceding the classic inclusion in tissue cultures of rabbit corneal cells has been presented (Scott *et al.*, 1953). Scott's latest studies (Gray and Scott, 1954) on the titration of the virus in different fractions of chick embryo liver cells again support the idea of localization within the nucleus. They propose that the necessity of fractionating early in the development of the lesion might explain previous failure to find nuclear localization (Francis and Kurtz, 1950).

C. Electron Microscopy

The clearest evidence for the intranuclear localization of the virus is the electron microscope study of Morgan *et al.* (1954a). In this study of a chick embryo-adapted strain of the virus, there is presented a series of beautiful pictures, shadowed and unshadowed, of clumps and masses of particles, most of which are found within the nucleus of the cell and which seem to have a characteristic virus-like appearance. The authors refer to three size classes of particles. The particles of the first class seem difficult to differentiate from normal cell components, but those of the second class, seen clearly in unshadowed material, are uniform in size and have a dense center (40–50 mμ) surrounded by a single membrane 70–100 mμ in diameter. The particles of the third class are formed in the cytoplasm; most of the particles show a double outer membrane 120 to 130 mμ in diameter. The authors suggest that there is a progression of development from the "primary" to the third stage.

The intermediate-sized particles in the shadowed material in Morgan's study are very similar to the "virus" particles found in 15 % of the tissue cultures of normal chick embryos (Gey and Bang, 1951; Bang, 1954). Since Morgan's strain of herpesvirus had been carried through a number of embryo passages since 1947, the possibility of contamination by chick embryo viruses needs to be considered. Grossly similar particles within the nucleus of nerve cells infected with herpes have, however, been recently described (Wyckoff *et al.*, 1956).

D. Herpes B

A related virus, herpes B, was studied by Riessig and Melnick (1955) in tissue cultures of monkey kidney, and the cellular changes were correlated

with measurements of the infectious virus produced. By infecting at a given time with an amount of virus sufficiently large to infect most of the cells, it was possible to follow sequentially the cellular changes. They found the same groups of particles with dense centers as described by Morgan *et al.* (1954a) but reported that they appeared in the cytoplasm and the nucleus at the same time. They classified them as (a) particles with a single membrane which ranged in size from 60 to 100 μ; (b) particles with two membranes (120 to 180 μ), which occurred both in the cytoplasm and the nucleus; and (c) particles about 180 μ in diameter which had a double central body. These last were found both in the cytoplasm and on the external surfaces of the cell. Some of the particles appeared to have two nuclei, each with its own membrane, the pair juxtaposed within a single outer membrane.

Although mitochondrial changes produced by herpes simplex have been briefly described in electron microscope studies of herpes-infected tissue (Bang, 1955), they have not been studied in detail, and knowledge concerning the effect of this virus on the cytoplasm is lacking. A study of differentially stained "inclusion bodies in experimental herpetic infections of rabbits" by Cowdry and Nicholson (1923) led to inconclusive results on this point. Yet this might be particularly important material, since the herpesvirus apparently travels long distances along the axon of a nerve cell as it progresses from cell to cell and leaves behind the characteristic nuclear lesion.

E. Fluorescent Antibody Staining

A study with fluorescent antibody by the double antibody technique on two strains of herpesvirus (one recently isolated and one "classical strain" in unfixed preparations of trypsinized tissue cultures of human amnion and infant mouse kidney cells) showed a specific peripheral fluorescence in these cells. Despite the demonstration of morphological changes at 18 hours, of intranuclear lesions at 24 hours, and cell degeneration at 31 hours, the fluorescent staining of the nucleus itself was not clear in any of these cultures (O'Dea and Dineen, 1957). This limited staining of the cell may, however, as the authors point out, have been due to "impermeability of unfixed cells" to the globulin. These results are contrasted to Lebrun (1956), who found virus antigen first as small foci within the nuclei of infected carcinoma cells in tissue culture. Later large fluorescent masses accumulated in the nucleus, followed finally by cytoplasmic accumulation of antigen. It was at this last stage that the classic intranuclear inclusion was apparent. Lebrun refers to this as an intranuclear scar. Large amounts of virus were used and thus, presumably, most of the cells were infected early.

VIII. Myxoviruses

A. Introduction

The influenza, Newcastle, mumps and fowl plague viruses have been recently classified together because of morphological and biochemical similarities (Andrewes *et al.*, 1955).

As with other animal viruses, very little is known about the penetration of these viruses into cells. Although phagocytosis may occur even in ciliated cells (Ropes, 1930), it may only be presumed that virus may gain entrance to the cell by this means. The consistently active pinocytosis in chicken macrophages was used to follow the entrance of Newcastle disease virus into these cells (Hotz and Bang, 1957b), but no details of the process were apparent in the electron microscope sections other than the presence of virus in the fluid droplets as they were engulfed. Perhaps because of the peculiar filamentous forms (Mosely and Wyckoff, 1946) which occur with some of the members, they have been studied more thoroughly than some of the other animal viruses. Although the generally destructive effect of influenza virus on certain respiratory cells was well known (Stuart-Harris and Francis, 1938; Perrin and Oliphant, 1940; Hers, 1954), no clear picture of the effect of these viruses on the cell was available before electron microscopy showed the more detailed changes present in cells. Recent studies with fluorescent antibody have also been particularly revealing. Phagocytosis of influenza virus by human and mouse leucocytes has been demonstrated by using the fluorescent antibody technique (Boand *et al.*, 1957), but this procedure has not been applied to normally susceptible cells.

However, as will be brought out in the discussion, the effects of these viruses may well vary with the strain of virus used and with the type of host cell. For this reason we have summarized in Table I the references to appropriate studies. The blank areas represent our lack of knowledge of any pertinent electron microscope studies. It may be seen clearly that although a number of different cell systems and some variation in virulence of the viruses have been studied, a preponderance of work has been done with the cells of the allantoic sac and relatively little with the cells which are most involved in the natural diseases.

Although it is possible to see influenza virus filaments by dark field microscopy (Chu *et al.*, 1949), and to stain them (Lindemann, 1957), the problem of their formation has incited a maximum of effort by electron microscopists. It seems clear that the clue lies in the surface activity of the cell, but the very superficial nature of this activity may hide the deeper problem of how the virus itself formed. This has been made particularly clear by the studies on the nuclear localization of antigen by fluorescent antibody technique.

TABLE I

ELECTRON MICROSCOPE STUDIES OF THE EFFECT OF MYXOVIRUSES ON CELLS

Virus	Chick tissue culture cells			Sections of cells in host		
	Epithelium	Fibroblast	Macrophages	Allantoic cells	Respiratory epithelium	Ascites tumors
M. influenza A Adapted	Murphy *et al.*, 1950; Murphy and Bang, 1952			Murphy and Bang, 1950; Morgan *et al.*, 1956a; Eddy and Wyckoff, 1950	Hotz and Bang, 1957a Harford *et al.*, 1955	
Unadapted				Morgan *et al.*, 1956a; Bang and Isaacs, 1957		
Swine	Murphy and Bang, 1952			Murphy and Bang, 1952		
M. pestis galli (Fowl plague)	Flewett and Challice, 1951			Hotz and Schaefer 1955		
M. multiforme Newcastle disease virus Virulent Avirulent	Bang, 1953a	Bang, 1953a	Hotz and Bang, 1957b	Bang, 1953b; Morgan and Wyckoff, 1950	Burnstein and Bang, 1958	Adams and Prince, 1957
M. parotidis (mumps)				Bang and Isaacs, 1957		

B. Filament Formation

Since more is known about the surface activity, however, we will discuss it first. The presence of a variable though usually small number of typical spherical forms of the virus along the filament and usually at the tip has been described by several authors (Murphy *et al.*, 1950; Bang and Isaacs, 1957; Wyckoff, 1953; Archetti, 1955) and early suggested an association between the two (Figs. 5, 6, 7). On the basis of an earlier observation, Robinow (1950) proposed that these filaments arose from the edge of the cell. There followed a series of observations on tissue cultures (see Table I) which showed that a profusion of filaments may occur when previously infected cells are grown in tissue culture, and that virus action was directly related to the surface of the cell. This relationship was established by tissue culture studies of both influenza (Murphy *et al.*, 1950; Murphy and Bang, 1952) and Newcastle disease virus (Bang, 1953a) (Figs. 8, 9, 10). Material taken at intervals following infection with three members of the myxovirus group—influenza (Bang and Isaacs, 1957; Morgan *et al.*, 1956a) Newcastle (Bang, 1953b) and fowl plague, and sectioned (Hotz and Schaefer, 1955) showed a profusion of filaments put forth at the surface of the cell during the later stages of infection. Major surface changes may occur without apparent change in the structure of the underlying cytoplasm. These cells normally have a number of microvilli on their surface (Borysko and Bang, 1953; Bang, 1955a; Morgan *et al.*, 1956a). This, combined with the fact that cells frequently form arrays of variable extrusions when damaged, suggested that this process was nonspecific (Hoyle, 1950). However, the presence of spherical morphological units within the filament, of red cell-agglutinating activity along the filament, and the great length and relatively uniform diameter of the filaments indicate a fair degree of specific viral activity in the filament (Bang and Isaacs, 1957). Recent enzymatic analysis of these filaments by Valentine and Isaacs (1957a) has shown that the filaments developed rows of spheres along the long axis when treated with acid and that these spheres were completely digested with trypsin. On the other hand, the separate spherical forms contained trypsin-resistant rings, tentatively identified as ribo-nucleoprotein (Valentine and Isaacs, 1957b).

Filament formation is particularly characteristic of cells infected with recently adapted strains of influenza, and very similar long cellular extrusions are found with infection with the mild or vaccine strain of Newcastle disease virus.

In contrast, the virulent strain of virus destroys the cell, and it is assumed that a virus is liberated as the cell boundaries disintegrate around it. The two distinctive devices by which avirulent and virulent viruses are released from a parasitized cell would inherently affect the pathogenesis of the disease in the host. However, it should be emphasized that no particles identifiable as

virus have been seen deep in the cell, whether the cell responds with a profusion of filaments or by complete disintegration.

There are numbers of studies of the effects of these viruses on both undifferentiated and specialized cells. It would be gratifying to be able to plot these in an orderly pattern which could be the key as to how and where virus is synthesized. Unfortunately, this again would be premature, for virus strains, cell types, and methods of study have all varied so much that generalizations based primarily on morphology would rather confuse than clarify the question. The remainder of this section therefore will review studies on particular virus species in relation to the method of study rather than in relation to the host cell.

C. Living Cells

Although the gross destructive effect of virus on certain cells is readily observed in tissue culture (Bang and Gey, 1951; Bankowski and Hyde, 1957; Tyrrell, 1955), there have been few studies on the living system either by the standard higher magnifications or by the improved resolution of phase microscopy. Exceptions are the study of Flewett and Challice on fowl plague (1951), in which phase microscopy clearly shows the development of nuclear masses which broke down as the cell disintegrated, and the study of Henle *et al.* (1954), who followed the formation of false giant cells infected with mumps virus and caused by an early lysis of the cytoplasm of small clumps of cells. The boundary between the cells then disappeared and only later did the nuclei disappear.

D. Fixed Tissues

The destructive effect of influenza virus on ciliated epithelium is well known. The virus, however, is not invariably destructive (Harford and Hamlin, 1952), and destruction, when it does occur, may be preceded or accompanied by certain cytoplasmic inclusions (Mount, 1951; Harford and Hamlin, 1952). The cytoplasmic inclusions in parotid glands with mumps (Johnson and Goodpasture, 1936) may be similar but also may be reaction to injection (Bloch, 1937). Thus, the recent studies of Wolff (1957) are important but difficult to relate to previous work. By microspectrophotometry of appropriately stained material (Feulgen for DNA and periodic acid Schiff's for glycoprotein), he was able to follow the amounts of these substances at intervals after infection. The mouse-adapted strain of influenza A(WS) consistently lowered the amount of glycoprotein in the cytoplasm of bronchial epithelial cells during the first 6 hours of infection. Active virus accomplished this, but heat-inactivated virus produced no change. The DNA content of these same cells was decreased with active infection during the first $7\frac{1}{2}$ hours and then

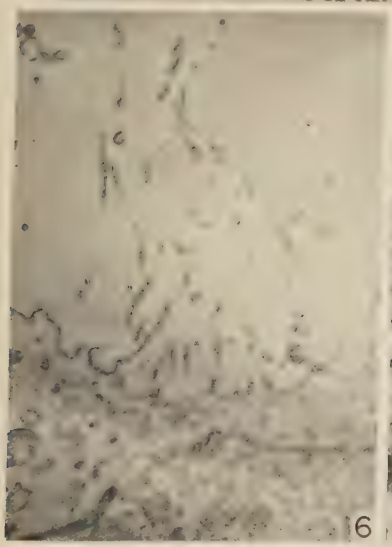
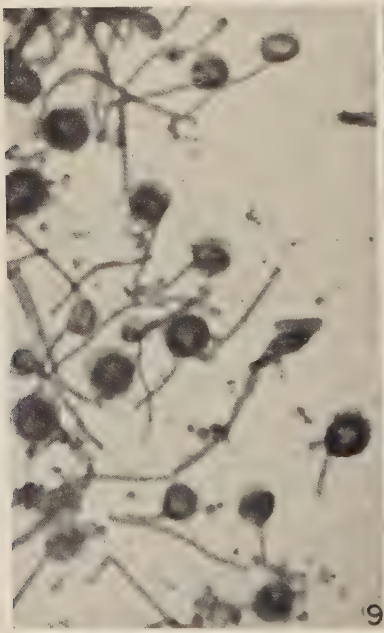


FIG. 5. Tissue culture preparation of influenza virus. A prime. Cells have been removed and residual virus is shown. Segmenting forms are shown in mid-left field. (From Murphy and Bang, 1952) (Magnification: × 7500).

FIG. 6. Section of membrane infected with recently adapted strain of influenza A virus. Long filaments project into the fluid. (From Bang and Isaacs, 1956) (Magnification: × 14,200).

FIG. 7. Section through 2-day-old infection with swine influenza virus. (From Murphy and Bang, 1952) (Magnification: × 15,000).



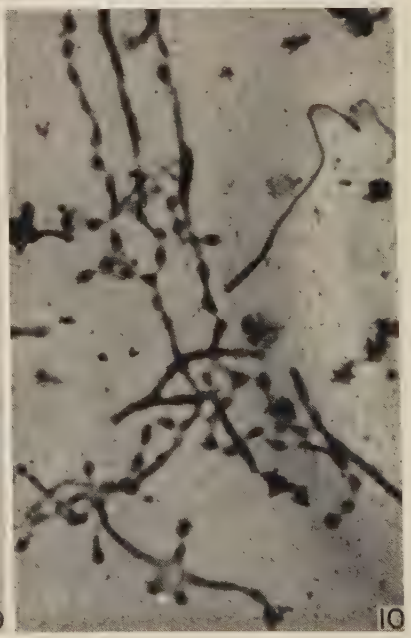


FIG. 8. Surface of chorioallantoic cell infected with Newcastle disease virus (mild strain) showing elongated microvilli with ballooning tips and unaltered cytoplasm. Dark large oval body in center is red cell apparently adherent to virus extrusions (Bang, 1955a) (Magnification: × 18,000).

FIG. 9. Newcastle virus infection of tissue culture showing dense spherical masses at tips of filament (Bang, 1953a) (Magnification: × 10,000).

FIG. 10. Newcastle virus infection of tissue culture. Long irregularly segmented and pinched filaments with roughly spherical forms along the filaments and extruded from them (Bang, 1953a) (Magnification: × 10,000).

was increased until it reached amounts exceeding that in the controls. Heat-inactivated virus caused a distinct increase in DNA during the first half hour of infection, after which the content returned to normal. As the author points out, these changes in DNA are hard to interpret in relation to virus synthesis, since the virus contains little or no DNA. They may be part of the cell's reaction to cytoplasmic events. The need for studies similar to this on other cell virus systems, particularly a type of tissue culture in which most of the cells can be infected simultaneously, is obvious.

E. Electron Microscopy

The first electron microscope study of respiratory epithelium infected with influenza is that of Harford *et al.* (1955), in which mouse bronchial epithelium was used. It is particularly valuable because it is a continuation of a careful study (Harford and Hamlin, 1952) of the same lesions by light microscopy. In both studies it is apparent that many ciliated cells persist at the height of the lesion and that certain intracytoplasmic inclusions are definitely present in the ciliated cells. An electron microscope study of the ferret mucosa (Hotz and Bang, 1957a), in which much more ciliated epithelium is destroyed, raises several comparative points. Since dead ciliated cells seem to be readily sloughed out into the lumen, or phagocytized, it is possible that a high proportion (even half) of the ciliated cells was destroyed and sloughed away early, leaving the adjacent, less-affected cells behind. Secondly, phagocytized material, particularly degenerating cytoplasm, would simulate in many ways the microscopic picture described by Harford *et al.* These questions would seem open until combined microspectrophotometric measurement of DNA, electron microscopy, and fluorescent antibody studies were all done in the same cell system.

The electron microscope study of ferret mucosa (Hotz and Bang, 1957a) infected with influenza showed details of cell destruction and disintegration which included a ballooning of the lamellar system of the cells (endoplasmic reticulum) and of the mitochondria, and the accumulation of fluid between the nuclear and cytoplasmic portions of the "nuclear" double membrane. The process of regeneration of cilia could be observed, but no specific virus effects were found.

F. Fluorescent Antibody Staining

This important new technique, developed by Coons and Kaplan for antigen localization, has been used to study the viruses of mumps (Watson, 1952), influenza (Watson and Coons, 1954), and Newcastle disease (Burnstein and Bang, 1958) in the chorioallantoic sac of the chick embryo. It has also been used by Liu (1955a,b) to study influenza in the ferret mucosa and human

nasal washings (Liu, 1956), and by others to follow the development of so-called incomplete virus in ascites tumor cells (Prince and Ginsberg, 1957).

1. *Mumps*

The first of these studies (Watson, 1952) on mumps in chick embryos showed that mumps antigen was present in those cells in contact with the amniotic fluid (embryos inoculated intra-amniotically), such as amniotic, epidermal, and pharyngeal epithelium. It was subsequently found in the gastrointestinal and respiratory tracts and in an acellular horny layer. The antigen appeared as bright intracytoplasmic granules. Electron microscopy of chick embryo allantoic infection with this virus shows surface changes only on the allantoic cells (Bang and Isaacs, 1957).

2. *Influenza*

Influenza virus (PR8 and LEE B) in the amniotic sac also apparently localized in cells lining the amnion, and in epidermal and pharyngeal epithelium. Specific staining appeared when the amniotic fluid contained 4.5 LD_{50} log of virus. Watson and Coons (1954) suggest that the antigen is first detectable in the nuclei or around the nuclear membrane, and that it then appears in the cytoplasm. In one pair of pictures, in which a slide previously stained with fluorescein was restained with hematoxylin, the nuclear or perinuclear localization of the antigen seems clear.

The histological localization of influenza virus in ferret epithelium is clearly shown by Liu (1955a), who was able to follow the progress of the infection, including desquamation of epithelium and uptake of viral antigen by macrophages. The presence of viral antigen was demonstrated in the cytoplasm and ciliated border of the epithelial cells. Several pictures show numerous, large, fluorescent, intracellular masses which appear to be the individual nuclei of the epithelial cells. In a separate study of this, Liu (1955b) finds that the fluorescence, which he believes to be nuclear, was responsible for the cross-fluorescent staining reactions among the three strains of A virus studied, and that absorption of the antisera with the V and S antigens indicated that the S antigen was responsible for the nuclear fluorescence. Since the large intracytoplasmic inclusions in the mouse bronchus were thought to be virus, and since ferret cells destroyed by influenza are apparently phagocytyzed by neighboring cells (Hotz and Bang, 1957a), the masses of fluorescent antigen need to be differentiated from possible phagocytized material.

3. *Fowl Plague*

The intranuclear localization of the "G antigen," or bound antigen of fowl plague, which is closely related to influenza, was determined by Breitenfeld

and Schaefer (1957). Following inoculation of embryonic chicken cells with sufficient virus to infect 90 % of the cells, the appearance of this antigen and the hemagglutinin antigen was followed. At 3 hours the bound antigen was present in the nucleus, whereas the hemagglutinin was found throughout the cell, with special concentration in the paranuclear area. Hemagglutinin antigen was found in the filaments.

G. Incomplete Virus

Morphological study of "incomplete" virus had, until the last year, been limited to observation in purified preparations (Werner and Schlesinger, 1954; Bang and Isaacs, 1957). The interaction between Newcastle disease virus and Ehrlich ascites tumors cells is in some ways comparable to the incomplete virus formation. Prince and Ginsberg (1957) showed that fluorescent staining detected the development of intracellular antigen, even when there was neither a rise of infectious titer, nor any demonstrable hemagglutinating or complement-fixing antigen. Since the intracellular antigen appeared in scattered cells even when the ratio of infecting particles to cells was less than one, it was assumed that the antigen increased within the cells. However, the antigen appeared only when the cell virus system was inoculated intraperitoneally in mice. A subsequent electron microscope study by Adams and Prince (1957) of this same cell system brings out the development of masses (apparently mostly paranuclear) in which a collection of small granules of about 3 to 14 mμ in diameter is surrounded by multiple lamella. The external portion of these lamellae are continuous with the endoplasmic reticulum. As suggested by the authors, this hypertrophied lamellar system may represent the fluorescent staining antigen. Further studies on other virus systems will probably reveal more lesions of this kind, but an interpretation of the events is not justifiable as yet.

IX. Adenoviruses

A. Cytochemistry

This newly discovered group of viruses, first uncovered because of their effect on human tissue cultures (Rowe *et al.*, 1953; Hilleman and Werner, 1954), has been studied almost exclusively in tissue culture as far as their cell lesions are concerned. Although the first analysis of their remarkable intranuclear lesions was with the electron microscope (Lagermolm *et al.*, 1957; Harford *et al.*, 1956; Morgan *et al.*, 1956b), we will review their effect as seen in stained, unsectioned material first. In a study of the sequential changes which occurred in HeLa cells infected with types 1 to 4, Boyer *et al.* (1957) found that types 1 and 2 could be differentiated from 3 and 4. In the first

two, rounded, Feulgen-negative, eosinophilic, intranuclear inclusions were found 14 to 16 hours after infection. Later, the nuclei enlarged and Feulgen-positive basophilic material appeared against a glassy nuclear background. With types 3 and 4, the lesions appeared at about the same time and appeared as sharply defined crystal-like masses which also varied in their reactions to the Feulgen stain. The nuclei enlarged and developed a "flower-like" appearance. Microspectrophotometry of these nuclei showed increased amounts of DNA in the infected cells. The cytoplasm was noted as disintegrating late in the development of the lesion. However, in none of the reported studies of the effect of the virus has an attempt been made to follow specific early changes with phase microscopy or vital stains. The early appearance of the strongly eosinophilic inclusion while the nucleolus remains intact and then the development of a late basophilic inclusion has been confirmed by Lépine *et al.* (1957).

B. Electron Microscopy

Three groups of investigators have described the remarkable intranuclear array of virus particles. The study of Lagermolm *et al.* (1957) was carried out with a series of cells in tissue cultures fixed from 6 to 72 hours after infection with $10^7 ID_{50}$ of virus. With type 5 virus, the appearance of small collections of particles, often adjacent to the nuclear membrane, at 24 hours corresponded with a slight rise in infectivity and the first appearance of cell lesions with the light microscope. By 48 hours of infection the nuclear changes were more regular and the crystal structures contained many more particles. At 72 hours some of the cells still did not show nuclear or cytoplasmic changes. In some there was a complete replacement of the nuclei by the virus particles, which were 30–60 mμ in diameter. It was estimated that with a cuboidal arrangement of these particles occupying about 100 cμ of crystal volume there would be approximately 10^6 virus particles per cell.

In their complete paper Harford *et al.* (1956) describe nuclear changes very similar to those mentioned above in types 1 to 4: the arrangement in rows, the presence of internal bodies within the particles, which measure 65 mμ at maximum size; the variable staining of the inclusions with Feulgen's reagent is again noted.

In a detailed study of the virus particles within the nucleus of HeLa cells infected with type 3 (4 and 7 were also studied but not presented), Morgan *et al.* (1956b) concentrated on the apparently regular variation in the density of the numerous particles as they occurred in the nuclei of otherwise intact cells. They propose that when the virus is packed into a crystal array the lattice is a cubic, body-centred packing of identical spheres. The regular alternation in density of the rows of particles in the crystalline array is explained by the fact that the thin sections of these arrays, which are of the

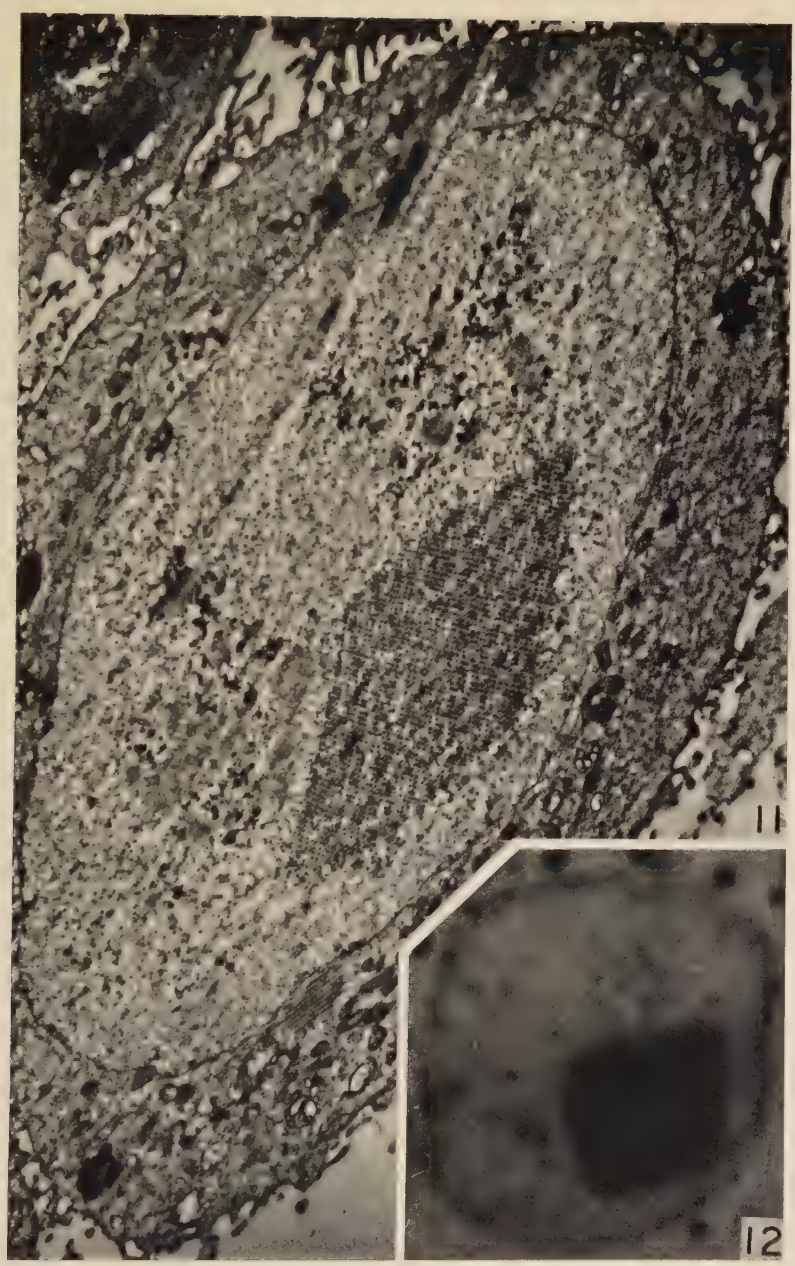
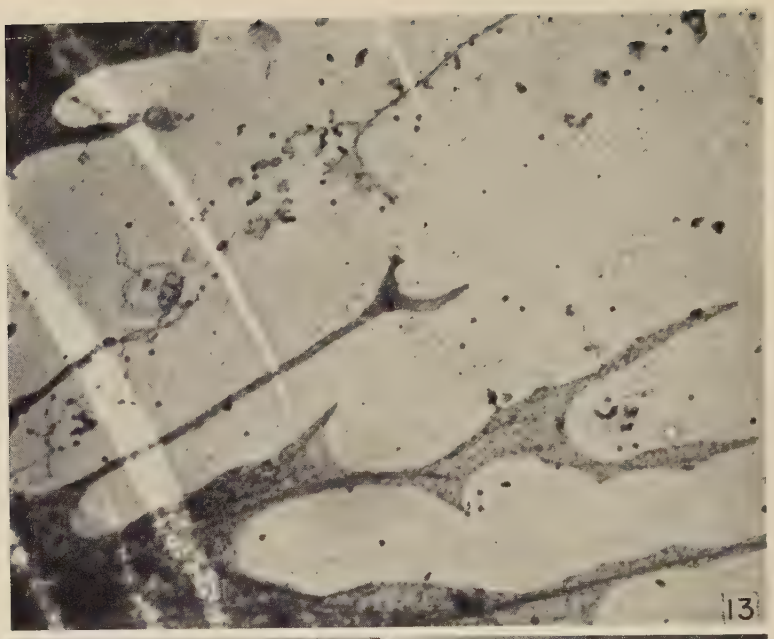


FIG. 11. Thin section showing well-formed crystal of rhomboidal profile in the nucleus of an infected cell. Adjacent to it are two masses of osmiophilic material in association with some scattered viral particles. (Courtesy Dr. Councilman Morgan, from *J. Biophys. Biochem. Cyt.* **3**, 1, 1957) (Magnification: × 9000).

FIG. 12. Contiguous thick section showing the same crystal which is strongly colored by the Feulgen reaction. (Courtesy Dr. Councilman Morgan, from *J. Biophys. Biochem. Cyt.* **3**, 1, 1957) (Magnification: × 3700).



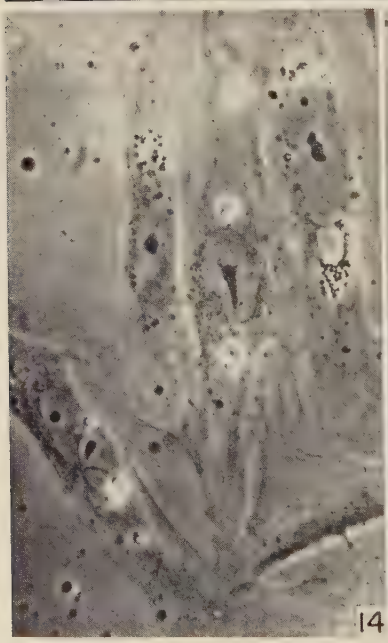


FIG. 13. Electron micrograph showing partial destruction of rat fibroblast by virus of eastern equine encephalomyelitis in tissue, culture (From Bang and Gey, 1952) (Magnification: × 20,000).

FIG. 14. Tissue culture of human sarcoma cells by phase microscopy. (Courtesy Stoler and Gey, 1953.)

FIG. 15. Tissue culture of cells from same line (AF) showing initial stages of cell destruction with poliomyelitis virus. (Courtesy Stoler and Gey, 1953.)

same general thickness as the virus particles themselves, would contain different proportions of the virus particles, dependent upon the amount of spherical virus caught in the particular section. Low and Pinnock (1956) have further analyzed these appearances and rule out other arrays as explaining the pictures obtained by Morgan *et al.* (1956b). Recent proposals by Valentine and Hopper (1957) that the true shape of the virus is polygonal (as seen in dried specimens), and that the shape is dependent upon the method of preparation (Tousimis and Hilleman, 1957) may have relevance to this characteristic crystalline pattern. Bloch *et al.* (1957) studied intranuclear lesions from the same nuclei in Feulgen-stained and in electron microscope preparations. By studying alternate thin and thick sections of HeLa cells infected with types 3, 4, and 7, the intranuclear Feulgen-positive masses were positively identified with the ordered arrays of virus particles (Figs. 11, 12). It was proposed that the virus particles developed from a Feulgen-negative matrix. It was further suggested that viral and host DNA may be differentiated by their reaction to a Feulgen azure-staining method. Finally, type 5 virus infection of another cell type (Hep 2) has been shown to contain an unusual crystalline protein within the infected nuclei. This new crystalline lattice consists of much smaller units and the complete inclusion is Feulgen-negative.

It is clear from all of these studies that an exciting beginning to the study of intracellular lesions with this virus has been made. The dynamic aspects will need not only sequential studies, but living cells and stained preparations must be analyzed with fluorescent antibody.

X. Poliomyelitis and Other Neurotropic Viruses

A. Nerve Cells

1. *Classic Findings*

Until the relatively recent evidence that a variety of tissue culture cells was susceptible to poliomyelitis (Enders, 1954), attention had centered on the lesions of the central nervous system and preeminently on the anterior horn cell. Although the pathology has been fully reviewed (Bodian, 1948; Howe, 1952), the main points will be recapitulated in comparison with lesions produced in the tissue culture system. The progressive changes begin with an initial massing of chromatin, continue with chromatolysis, which progresses even while mitochondria remain intact (McCann, 1948), and proceed eventually to complete necrosis of the cell and subsequent variable neuronophagia. Of particular interest now are some samples of a motor nerve cell lesion which was occasionally present after the acute stage of paralysis (Bodian, 1948). This lesion comprised a large eosinophilic mass, sometimes with the nucleus

"apparently verging on extrusion from the cytoplasm." In addition to actual destruction of the nucleus in the terminal stage of the infection, small eosinophilic intranuclear inclusions were sporadically found.

Electron microscope studies of cells of the central nervous system infected with poliomyelitis have not been reported. However, the identification of the Nissl substance which is first destroyed by virus as endoplasmic reticulum (Palay and Palade, 1955) and the presence of quantities of this material at the tips of the dendrites adjoining axons (Palay, 1956) would suggest that the virus is able to parasitize susceptible material throughout the cytoplasm of these specialized cells.

2. *Tissue Culture*

Living nerve cells have been studied in relation to the effects of the virus upon them in tissue culture. In all three types of virus (Hogue *et al.*, 1955, 1958), the first identifiable lesion was contraction and disintegration of the extended dendrites. Contraction began at the tips of these long processes and progressed until a process had withdrawn, at times leaving a bulbous extrusion. Later the cell became granular, lost its surface film, and assumed the character of a loose mass of granules surrounding the nucleus. Serial photographs demonstrate these changes. Although pinocytosis has been seen to occur at the tips of the axons in nerve cells in tissue culture (Hughes, 1953), the role of this event in susceptibility to virus has not been considered.

B. Epithelial Cells and Fibroblasts

There have been many careful studies of the effect of poliomyelitis virus on tissue culture cells other than nerve cells. These include phase (Stoler and Gey, 1953; Barski *et al.*, 1955; Riessig *et al.*, 1956; Klöne, 1955a; b) and bright field (Harding *et al.*, 1956) studies on living cells (Figs. 14, 15). There have also been several sequential studies of such changes in which cells were stained and fixed in correlation with the time of virus release (Riessig *et al.*, 1956; Dunnebacke, 1956a).

1. *Morphological Changes Observed in Living Cells*

The sequence of changes has been studied in individual cells by phase microscopy. Klöne (1955a) followed the effect of virus on monkey kidney cells which showed no lesions 23 hours after infection. These retracted from surrounding cells at 25½ hours, the nucleolus lost form, and protoplasmic extrusions appeared. Mitochondria remained normal through all of this activity. At 26½ hours the cellular contraction continued, and the sharply defined, irregular cell extrusions progressed. By 27½ hours the extrusions developed a series of fine extensions and branches, the cell nucleus was only obscurely visible, and vacuoles appeared in the cytoplasm. In the course of a

few hours, therefore, changes in the nucleus and cytoplasm appeared almost simultaneously. In comparing these findings with the data on fixed and stained cells, it is important to remember that only extremely careful cytological preparation would preserve intact the irregular peripheral spread of endoplasm so that it could be identified in fixed and stained material.

Barski *et al.* (1955) recorded the changes produced in large fibroblasts derived from tonsils in motion pictures taken by phase microscopy and described the development of large eosinophilic paranuclear masses. These occurred with both types 1 and 2 in cultures kept at 30 to 31°C. for 20 hours after the addition of a virus. The cell remained motile, and filamentous mitochondria persisted. Later, a series of bubbling extrusions developed, and the authors suggest that this is the mechanism of virus release. Very similar, if not identical, paranuclear lesions have been described in anterior horn cells by Bodian (1948); it is difficult to differentiate this lesion from the giant centrosphere described by Lewis (1920) in degenerating mesenchyme and in tumor cells. It may represent an abnormal accumulation of normal cellular material, rather than a specific virus "inclusion." The material has been shown to be Feulgen-negative (Harding *et al.*, 1956).

2. *Relation to Virus Release*

The exact relation of these cell changes to the time of virus release has not been settled. In studies on isolated individual cells, Lwoff *et al.* (1955) showed that about one hour before the beginning of virus release into the medium the cell started to contract, and a hyaline zone developed at the periphery of the cell. At the time of virus release this zone became vacuolized and then disintegrated, by which time virus release ceased.

Riessig *et al.* (1956) have followed the changes in cultures of monkey kidney cells during one phase of virus multiplication. Most of the preparations utilized hematoxylin, eosin, and Feulgen stains. Osmium fixation was not used. Some of the living cells were studied by phase microscopy. Although gross changes were not apparent in the infected test tubes until 8 hours after infection, the stained preparations showed a patchy disappearance of the chromatin network of the nucleus and development of an eosinophilic cytoplasmic mass by 5 hours, with the persistence of the nucleolus. The formation of intracellular virus began at about 4 hours, and extracellular virus increased from 5 hours on. The authors suggest that the changes in the appearance of the cell seen with phase microscopy do not begin until 1 or 2 hours after intracellular virus increases.

In studies on monkey kidney, HeLa, and human fetal cells, Dunnebacke (1956a,b) found that virus release occurred several hours after nuclear pyknosis, contraction of cytoplasm, and rounding of the cell. In contrast, human amniotic cells released virus much later, and the lesion in these cells,

as contrasted with monkey kidney, started with a disappearance of the nucleolus (Dunnebacke 1956b, 1957).

An interesting attempt to segregate the process of viral synthesis from the changes seen in the cell used the metabolic inhibitor, fluorphenylalanine (Ackerman *et al.*, 1954). Apparently, once the virus infection was initiated, although actual production of infectious virus was inhibited, cell changes proceeded. The changes described are mainly terminal, however, and a more detailed comparison of the viral growth and the cell changes in the same cultures is needed.

3. *Other Findings*

An interesting attempt was made by Klöne (1955b) to determine whether infection of monkey kidney cells had any direct effect on mitotic activity. No increase or decrease in mitosis rate was observed, but certain findings suggested that cells might be infected before mitosis set in, and that division would then proceed with eventual death of both daughter cells. By following individual cells for a number of hours, he was able to show that both daughter cells were destroyed at similar but not identical times. In one case, a cell was followed through to an abnormal telephase, from which the cell did not recover but fell apart.

In an electron microscope study of "virus-like bodies" in the nuclei of epithelial cells infected with type 1 poliomyelitis virus, Ruska *et al.* (1956) have reported on the occurrence of accumulation of particles of about 26 mμ in size, in close association with remnants of nucleoli. The occurrence of these particles, which in themselves are not highly distinctive, needs to be correlated with the appearance of infectious virus and the sequence of cellular changes.

The localization of poliomyelitis antigen by fluorescent antibody (Buckley, 1956) shows that type 1 may be found both in the nucleus and the cytoplasm and may be found in peripheral blebs of the cell as it breaks down. In the early stages specific fluorescence was diffuse or granular.

A summary of concurrent findings on the effects of poliomyelitis virus on living cells in tissue culture would include: (1) Peripheral contraction of the thin endoplasmic spread, which leaves ghosts of branched material behind to simulate irregular extrusions; (2) disappearance of the nucleolus; (3) formation of a large, paranuclear mass.

It seems clear that the morphological steps which lead to final death and disintegration of the infected cell cannot as yet be set in proper sequence. The cells vary in their response to the virus so that, as yet, early cytoplasmic changes have not been differentiated from nuclear destruction. Undoubtedly, part of the difficulty is that different cells react in different ways to the same virus (Dunnebacke, 1957). Thus, morphological evidence does not tell us

whether virus may be multiplying in the cytoplasm or the nucleus. The mitochondria, however, seem both in the nerve cell and tissue culture to remain unaffected until fairly late in the destruction.

C. Encephalitis

Although there have been relatively few cytological studies of the effect of the arbor (arthropod-borne) viruses on cells, these do illustrate certain general problems and so will be discussed here. The virus of eastern equine encephalomyelitis produced varying degrees of gross destruction of cells in tissue culture, dependent upon the cell strain used (Bang and Gey, 1952; Bang *et al.*, 1957). Using a tissue culture method of preparing cells for electron microscopy, the cytological aspects of these infections were studied. It was shown (Bang and Gey, 1952) that piecemeal destruction of the cell occurred. Individual fibrillar processes (Fig. 13), normal for the rat fibroblast, were destroyed by the virus before any effect on the rest of the cell was apparent. In other cells, the virus was found on the very edge of the cell, sometimes in the presence of destruction, sometimes without any change. Subsequent quantitative studies by Dulbecco of virus release from cells showed that this virus is gradually released from cells over a period of some hours.

This picture of varying or limited amounts of destruction is completely changed when the same virus is studied in primary explants of chick embryos, either in roller tube cultures (Bang, 1955a) or in slide cultures (Bang and Gey, 1949). Here, the destruction was shown to be accompanied by an almost complete replacement by the virus.

A somewhat related virus (Egypt 101) has been studied by the fluorescent antibody technique not only in mouse brain but also in cultures of human epidermoid carcinoma (Noyes, 1955). The antigen was found 24 hours after infection in the cytoplasm, but there was no nuclear staining. There was some concentration of the antigen in the paranuclear area at first, but later it was found distributed throughout the cytoplasm.

XI. Tumor Viruses

Three fundamental questions may be raised concerning tumors induced by viruses:

1. Are cells which are infected by neoplastic viruses morphologically different from cells infected with other viruses? Specifically, are there differences in the localization of the virus, the alterations in ultrastructure in the cell, the mitotic abnormalities, and the mechanisms of virus multiplication and virus release?

2. Is there any direct relationship between the presence of morphologically identifiable virus and the malignant nature of the cell? Are there reliable

controls, is there morphological evidence of lysogeny and/or latency, and is the continued presence of virus demonstrable throughout the existence of the tumor?

3. Is the specificity ascribed to some tumor viruses reflected in analogous specific effects in tissue culture? This question remains unsettled, even with respect to the well-studied Rous chicken tumor virus. Although the answers to these questions cannot yet be conclusive, it is encouraging that it is now feasible to ask them, and that the implements for finding the answers are available. The five selected types of tumors will emphasize the variety of cellular reactions to the viruses. This variety of reactions should not, however, obscure the fact that data on the above questions are not complete for any one virus-host cell system relationship.

A. Rous Chicken Sarcoma

Although this tumor was proved to be of viral etiology almost 50 years ago (Rous, 1911), there is a present need to understand the effect of this virus on living cells of established strains. Many early observations on living cells have been obscured by controversy over the nature of the tumor cell, whether it is macrophage or fibroblast. It is ticklish today to describe with assurance the effect of the virus on macrophages and fibroblasts, for it has been proposed both that the virus could convert macrophages into fibroblasts (Carrel and Ebeling, 1926), and that under other virus-induced conditions fibroblasts could become phagocytic (Fischer and Laser, 1927). Some light may have been shed on the enigma by the study of Sanford *et al.* (1952), who showed that fibroblasts grown in horse sera would support growth of the virus for six months, while macrophages failed to support it after a few days. There is no record of a comparable experiment using chicken sera.

Borel (1926) showed that cultures of the Rous tumor consisted of two types of cell: round basophilic cells and fusiform fibroblasts. The macrophages often yielded multinucleated plasmodia which at times reached a diameter of 600 μ. The fusiform cells were occasionally equally large. Both types of cells showed an accumulation of eosinophilic material in the cytoplasm corresponding to the eosinophilic paranuclear mass seen in the tumor cells in the animal. Hypertrophied nuclei and an accumulation of fat droplets were characteristic, as was an extraordinarily lavish network of mitochondria.

1. *Effect on Cells in Tissue Culture*

The most detailed study of these cells as cultivated *in vitro* is that of Doljanski and Tenenbaum (1942; Tenenbaum and Doljanski, 1943). They documented and fully illustrated the "distinctive syndrome of severe cell disease." Although their work was carried out on an "18-year old strain of the tumor," it was maintained with the regular addition of fragments of

normal tissue. Thus, the cell types themselves cannot be evaluated. In spite of this factor, the variety of lesions and the detailed photographic accounting of them are unsurpassed. These authors not only confirmed Borel's findings but described lesions of the nuclei in the basophilic cells in which an aggregation of the chromatin was seen within a decidedly granular nucleus. Some of the nucleoli assumed peculiar shapes or were pushed to one side by eosinophilic masses negative to Feulgen stain. Occasional nuclei were packed with eosinophilic inclusions. The marking-off of the central region of the cytoplasm was accompanied by the formation of intracellular crystals. Atypical mitoses were common as was giant cell formation, and chromosome lag during mitoses was illustrated.

A phase microscopic study (Lo *et al.*, 1955) of living cultured fibroblasts in non-chicken media and inoculated with the Rous virus showed that normal fibroblasts were transformed into abnormal cells which developed typical paranuclear inclusions and fatty accumulations. Giant cells formed again with great networks of normal appearing mitochondria.

2. *Electron Microscopy*

Since the first cautious description of "small bodies, the size of that estimated for the transmitting tumor agents," having the appearance of extraneous entities within chicken tumor cells in tissue culture (Claude *et al.*, 1947), there has been a series of descriptions of these particles both in tissue cultures (Bernhard *et al.*, 1953; Epstein, 1956) and in sections of tumors (Gaylord, 1955; Bernhard *et al.*, 1956a).

The identification of these particles as the tumor agent is as yet incomplete, but several of the criteria (Bang, 1955b) for such identification are gradually being fulfilled. The particles from the first were recognized as having an internal area of high density and a peripheral area of slight density. Recent pictures show an external membrane and a thinner internal membrane between the dense central portion and the external membrane (Bernhard *et al.*, 1956a). Thus, there is a fairly characteristic arrangement or grouping of the particles and an internal morphology for the individual particles. It is much more difficult, however, to make a clear statement relating these characteristic particles to the infectiousness of the material. Epstein (1956) was able to show a correlation between the percentage of cells showing these particles in vacuoles and the infectiousness of the extract. Whether these round and vacuolated cells obtained from ascites-like passages of the Rous tumor are indeed the tumor cells or are infected macrophages like those originally described by Borel is not pertinent to the identification of the particles. However, it is clear that there is a correlation between (a) the cells which contain particles and (b) the infectiousness of the suspension; and thus, indirectly with the particles. A more direct comparison of the

number of particles seen in sections of tumors and the infectiousness of the tumor extract has recently been completed (Hagenau *et al.*, 1958). In this study the more infectious tumors showed the greatest number of characteristic particles, and the pellets obtained from highly infectious extracts showed many more particles than those of low infectiousness. It is clear that a beginning in the identification has been made. However, the immunological tests, purification, spray droplet correlations, etc., which may conclusively establish these particles as the agents of infection have not been completed. Confirmation is especially important. A small percentage of normal chick embryo tissue cultures yield preparations with large numbers of identical particles (Bang, 1954; Gey and Bang, 1951).

Further complications are added when related tumors, such as the Murray-Begg endothelioma (Rouiller *et al.*, 1956) and leukemias (Benedetti *et al.*, 1956), are studied for both of these have similar particles. In the latter, 3 of 24 spleens from "normal" chickens showed the same particles. The morphological difficulties have then emphasized the fact that most stocks of chickens acquire antibodies to these agents (Andrewes, 1939; Bang and Haley, 1958), and that uncontaminated control animals are difficult to obtain. It is tempting to explain these contradictions by supposing that virus tumor cells carry the virus in a latent lysogenic state, and that the infectious and visible form of the virus is important in producing what may well be secondary pathological changes in infected cells, whereas the really malignant cell does not necessarily carry the virus in this form. The recognition that the disturbed cell is one in which host cell and virus are not in balance, whereas the transmissible tumor cell is in balance with its virus would support this idea (Bang, 1955a).

3. *Localization of Virus*

In all of the above studies on the Rous sarcoma the viruses have been found predominantly at the surface of the cell or within vacuoles which are in a way not in contact with the cell. It is, therefore, not easy to perceive the method of formation of the complete virus. In a few cases particles have aggregated within vesicles which could well be altered mitochondria.

The possibility that the characteristic virus particle, which is assumed to be the infectious unit, represents only a terminal phase of the partnership which is the tumor cell, focuses particular interest on the morphology of the tumor cell. Early observations on this are presented in the beginning of this chapter. Recent electron microscope studies of the "fibroblast-like tumor cells of Rous sarcomata" (Epstein, 1957) show that the ultrastructure of these cells is similar to that of other cells and fails to bring out any qualitative morphological difference between normal fibroblasts and the tumor cells. Attention was, however, directed upon the tightly packed piles of smooth

cisternae. These were found in two separate areas of the tumor cell, whereas they are normally found in only one area. These may represent part of the story of the hypertrophied paranuclear area.

B. Mammary Tumors of Mice

Although this tumor may now be listed among those associated with viruses, the biological data establishing this point are more complex than those available for the chicken tumors. The milk agent operates within a more restricted set of conditions (sufficient hormonal stimulus and a particular genetic background are essential) and the tumor itself has an extremely delayed incubation period. Secondly, the spontaneous mammary tumors which occur in low incidence in mice in the absence of "the milk factor" nevertheless have, in many cases, a histological structure indistinguishable from milk factor tumors (Dunn, 1953). In the continuing search for the nature of the cancer cell attention has repeatedly focused on variations in neoplastic cell structure. Nevertheless, the behavior of a group of cells has remained more diagnostic than the appearance of any one cell. For this reason, the recent study of Foulds (1956) is of particular value in orientation for a detailed study of individual cells. The restricted portion of tissue which can be studied in the electron microscope field has so far prevented adequate correlation of ultrastructure with the distribution of the developing malignancy.

1. *"Inclusion" and Virus Particles*

Eosinophilic cytoplasmic inclusions were described by Guerin in 1955 in several lines of mammary tumors. Almost at the same time, studies with the higher resolution of electron microscopy (Bang and Andervont, 1953; Bang *et al.*, 1956a,b; Bernhard *et al.*, 1956b; Dmochowski, 1954) showed that masses of intracytoplasmic particles thought to be virus could be commonly found in mammary tumor cells. Direct comparison of the cytoplasmic inclusions (Bernhard *et al.*, 1956b) and the eosinophilic inclusions showed that a large part, if not the entire mass, is made up of such particles. The first description of these particles is without question accreditable to Porter and Thomson (1948), who found clusters of these particles with an average outside diameter of 130 mμ and a dense center averaging 75 mμ in epithelial cells grown in tissue cultures from spontaneous mammary tumors. They were found in three of the six tumors studied in this way. It is likely that these "virus-like bodies" actually were at the surface of the cell, although several clusters which appear to be within the osmium-digested cell are also shown.

Sections of infected cells, however, have the great advantage of affording accurate localization of the particles. Remarkably similar pictures were

obtained by three separate groups of investigators (Bang, 1955b). Two types of particles were found and may be interpreted as representing two phases of development. One type was seen in paranuclear clusters of spherical (oval as compressed in sectioning) particles, having an outside diameter of about 65 to 70 mμ. The particles were also scattered throughout the cytoplasm and sometimes encircled small vesicles like a series of beads. They did not appear to have any specific relation to intact mitochondria, but it is possible that some of these small vesicles were remnants of degenerating mitochondria. Pictures suggestive of this have been published (Bang *et al.*, 1956a,b). Bernhard *et al.*, (1956b) has shown that this type of particle is actually double-layered, with an internal diameter of about 38–50 mμ and an external, sometimes less dense layer of about 10 mμ.

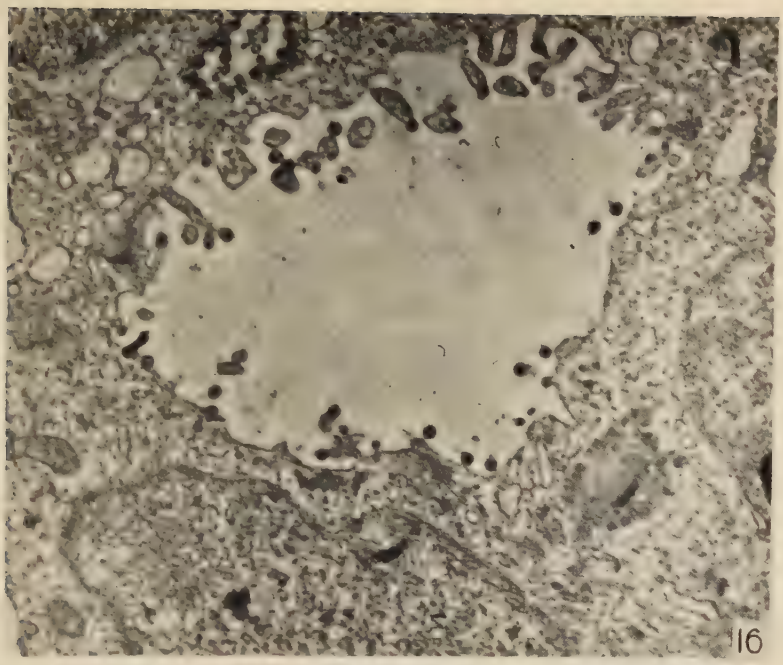
2. *Morphological Evidence of Virus Release*

The second group of particles is larger, was found either within vesicles, on the surface of the cell, or attached to the microvilli of the cell. The possibility that they are formed by the ejection of the smaller particle either from microvilli (Bang, 1955a; Bang *et al.*, 1956a,b; Bernhard *et al.*, 1956b) or from the free surfaces of the cell follows the pattern of virus release in other cell infections (Figs. 16 and 17). Their presence as dense particles surrounded by less dense "cytoplasm," yet still within the cell, might result from the projection of finger-like extrusions of the host cell cytoplasm into an intracellular vesicle which was the residuum of a collapsed mitochondrial structure. There is, however, no evidence of selective localization in or near mitochondria.

Serial sections have shown that they are released all along the free surface of a cell and that they may also project by a series of microvilli into fluid pockets between cells (Bang *et al.*, 1956a,b). The particles may be readily distinguished from the larger, more homogeneous milk particles or droplets the secretion of which entails a continuous breakdown of the cell surface (Bang *et al.*, 1956a,b; Bernhard *et al.*, 1956b).

Except for one preliminary, unconfirmed report (Kinosita *et al.*, 1953), there is no record of their presence in the nucleus of the host cell.

Since identical particles have been found by three different groups in a number of mammary milk factor tumors and rarely, if ever, in normal cells, and since their characteristic shape, arrangement, and apparent release from the cell surface follow those of other viruses, they are the most promising candidates for identification as the virus of mammary tumors. However, present biological knowledge of the virus is limited to factors known to be transmissible by milk and possibly by seminal fluid. Again, there is agreement (Bang *et al.*, 1956a,b; Bernhard *et al.*, 1956b; Dmochowski *et al.*, 1955) that identical particles are found in mammary tumors which lack the milk factor. Either these particles do not represent the milk factor or they may



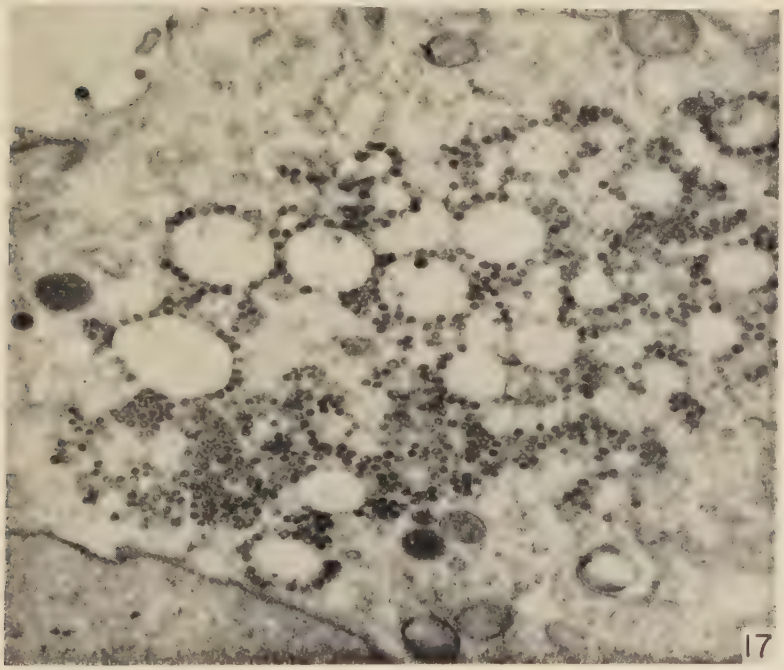
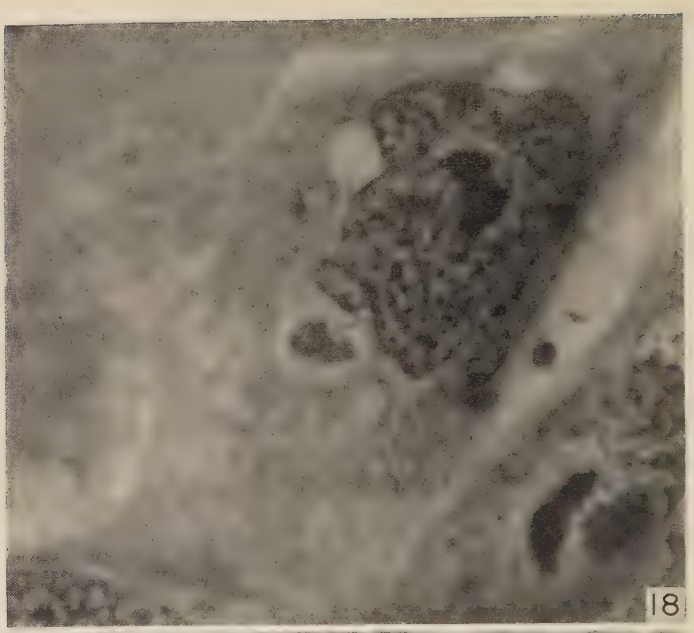


FIG. 16. Vesicle between two mammary tumor cells. Clump of early particles in right lower area of cytoplasm. Microvilli with virus particles within them projecting into vesicle. (From Bang *et al.*, 1956a) (Magnification: × 18,000).

FIG. 17. Collection of virus particles in cytoplasm of mammary tumor of mice. Two projecting microvilli are seen in upper left area. (Courtesy Dr. W. Bernhard: Bernhard, Guerin, and Oberling, 1956b.)



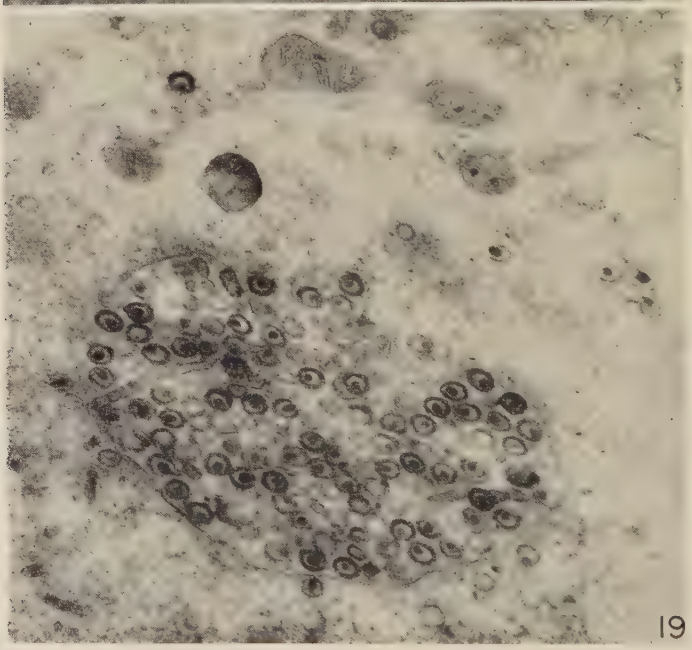


FIG. 18. Tissue culture preparation of frog adenocarcinoma with cytoplastic inclusion and lobulated nucleus. (Courtesy Dr. W. Duryee.)

FIG. 19. Thin section of frog adenocarcinoma showing mature virus particles within cytoplasmic vesicles. Complex structure of mature virus particles apparent. (Courtesy Dr. D. Fawcett, 1956.)

just look the same and may be equally essential to the mammary cell but spread from mouse to mouse by a route other than the milk. Further complexity was created when Fawcett and Wilson (1955) found somewhat larger particles in hepatoma cells from the mice (milk factor C_3 H mice) which commonly develop mammary tumors. Most of the work reviewed here on the mammary tumor agent has been concerned with the comparative morphology of tumor cells and normal cells. There is such a dearth of knowledge about living cells that Lasfargues's (1957a) cultures of adult normal and malignant mouse mammary cells are of particular interest. His observation of particles resembling those seen in the sections but found at the surface of the tissue culture cells (Lasfargues, 1957b) adds to the potential importance of this method of study.

C. Warts (Human Papillomas)

The viral etiology of these persistent benign growths has been indicated for a number of years (van Rooyen and Rhodes, 1948). In any common clinical sample of warts a few may be observed to differ from their neighbors at the same sites in that they have a smooth margin, less keratinization, and a surrounding erythematous inflammatory halo (Bunting *et al.*, 1952). Water extracts of such warts were shown to have masses of small, round, uniform particles which were often in a crystalline array (Straus *et al.*, 1949). Bunting showed that these apparent virus particles were closely packed within the nuclei of affected cells and in such crowded conditions measured about 38 mμ in diameter. In the cells of the Malpighian layer of the neoplastic epidermis, the particles were in the nucleus and did not occur in the prominent eosinophilic intranuclear inclusions or in the cytoplasm which contained characteristic discrete dark masses. In cells forming the lower stratum corneum of the wart, at a time when the nucleus was no longer recognizable, the particles occupied almost the entire cell (Bunting, 1953a). Bunting (1953b) later suggested that "apparently as the inclusion body becomes larger, it becomes granular and then becomes 'converted' into virus particles. These then increase in number and fill the nucleus. At first they are not in an array, but when the number is so great as to distend the nucleus when they are tightly packed then the array appears." Investigation of these papillomas was suspended upon Bunting's untimely death, but the similarity of these lesions with the changes wrought by some strains of adenovirus is striking.

D. Frog Adenocarcinoma

Since the incidence of spontaneous carcinoma of the kidney of frogs may be greatly increased by the injection of various filtrates from such tumors,

and since intranuclear inclusions occur in these tumor cells (Lucké, 1939), it is generally accepted that this tumor is caused by a filterable agent. Since investigation of the agent is still preliminary, this discussion will be limited to the cytological changes which differentiate the tumor cells from normal.

The recent much-needed tissue culture studies of Duryeé (1956) have brought us much closer to realizing a method by which a known number of virus particles may be placed on a known number of cells so that the morphological events involved in a one-step growth curve may be followed. The changes, which he has labeled as precancerous, may then eventually be quite precisely determined.

Under less exact conditions, Duryeé (1956) has studied the following changes produced by this virus in living cells: (1) Large irregular nucleoli, which often showed pulsations. (2) Large cytoplasmic inclusions, which were thought to originate in the nucleus, and were seen passively to extrude through the nuclear membrane. (3) Giant cells with 3 to 55 nuclei per cell. In addition to these direct observations, it was determined by means of a microdissector that the intranuclear inclusions were semisolid, gelatinous masses (Fig. 18).

An electron microscope study of these tumors by Fawcett (1956) has revealed numerous "virus particles" throughout these cells in about one-third of the tumors examined. These are hollow spheres (90–100 mμ) with a thick capsule and a dense inner body (35–40 mμ). These particles were found in the cytoplasm, occasionally in the nucleus, and in the microvilli, where they were presumably being extruded into the extracellular spaces (Fig. 19).

The intranuclear inclusion bodies which are described above were found to be largely made up of hollow spherical vesicles with a thin limiting membrane. These are thought to be "immature virus particles." A few of these contain a dense inner body like the "mature" cytoplasmic particles. Bundles of dense filaments and vacuoles, which were also found in the infected cells, were not explained. A sequence of development of the virus is suggested, but, again, is completely dependent upon hypothetical order of events.

In summarizing the distribution of virus particles in the different virus tumors we have included data on the Shope fibroma, even though it is reviewed in another section. It is evident that the tumor viruses, like other viruses, are found throughout the cell. Moreover, there is nothing distinctive about the type of cell pathology produced by them. This lends credence to the idea that these tumor cells in which virus is found and changes are produced are the cells which are out of balance with their parasitic virus. Thus, the balanced state, which indeed may be one analogous to that of the lysogenic bacteria, has not as yet been technically distinguishable from the normal cell.

XII. Discussion and Conclusions

In summarizing the experimental data on virus infections no attempt will be made to systematize contemporary knowledge of individual points but rather to collate some of the ideas and trends which are reorienting and even reorganizing research in this area.

1. It is axiomatic that a morphological study of virus infections involves the interaction of virus and cell. In this context the pathology of the cell may be expressed as virus action, cell reaction, and subsequent interaction until the cell overcomes or is destroyed by the virus. Given, then, the pathological state, the first necessity is identification of the virus, and for this there is no set of rules. Criteria for the identification of viruses in the electron microscope have been tabulated and discussed elsewhere (Bang, 1955b). These criteria have been generally fulfilled in the case of the poxviruses and perhaps in several others. Viral antigen may now be identified within the cell by the fluorescent antibody technique (Coons, 1957), and the application of this technique will no doubt be greatly expanded. The determination of that part of cell pathology which is a reaction to the virus is more difficult. Similar changes which are known to occur under various unfavorable conditions have perhaps been taken too little into account. An excellent example is the hypertrophied paranuclear area in infection with poliomyelitis, which is simulated if not duplicated in degenerating mesenchyme and in a variety of tumor cells.

2. The pathology of the cell now implies, not only the reaction of the entire cell, but also lesions of parts of the cell independent of apparent simultaneous change elsewhere within it. The adenoviruses are identified as masses of virus particles inside the nuclei of otherwise intact cells. The mitochondria in infections with poliomyelitis or Rous sarcoma virus remain intact until late in the extensive cellular changes which entail the accumulation of paranuclear material and, in poliomyelitis, the onset of nuclear destruction. Great growth of abnormal microvilli, which seem to have virus within them, develop at the surface of a cell infected with influenza or Newcastle disease virus, and yet the mitochondria are normal even at high resolutions in the electron microscope. Thus, we are beginning to get away from the term "inclusion," and we have avoided it here except when used in an established framework. It would be more accurate today to speak of lesions within the cell and to describe them as virus, virus by-products, or reactions on the part of the cell to the virus.

3. The ways in which a cell can react to a virus are probably limited, and the same reactive processes may be induced under a variety of conditions. The microvilli, which are normal extrusions from epithelial cells, seem to contain and, in some cases, to release virus in vaccinia, influenza, Newcastle disease, and frog adenocarcinoma. Even rickettsia may be extruded from the

cell by means of long fibrils. The intracellular fluid vacuoles, which develop in tissues infected with the larger viruses, may be considered as a way of separating parasite from host cytoplasm. The loss of osmotic competence of the cell is reflected in ballooning of the internal membrane system (endoplasmic reticulum) and the development of fluid vacuoles between the nucleus and the cytoplasm.

4. A series of electron microscope studies, particularly with the myxoviruses, has focused interest on the changes occurring at the cell surface. From this we have inferred that virus release, and perhaps maximum virus activity, occurs here. These data tell us nothing about where the various parts of the virus are synthesized. Indeed, interpretation of some morphological data on herpes has suggested that the early virus forms are made in the nucleus and that they acquire more complete clothing as they progress from nucleus to cytoplasm to the surface of the cell. The conclusiveness of a given interpretation may be hazardous, as witness the fact that two strains of the same virus may produce different cellular lesions (Bang, 1953b; Bankowski and Hyde, 1957), contingent upon their respective virulence. Furthermore, the same virus may produce different cellular lesions in different cell types. This latter circumstance has been nicely shown in the infection of rabbit fibroblasts and epithelium by the virus of myxoma by Chapronniere (1957). These may be compared with macrophages (Maral, 1957). It is also apparent in the effect of poliomyelitis on different cell types (Dunnebacke, 1956a,b).

5. There is a great need for correlative studies of one virus on one cell system by different methods, with concomitant correlations of the amount of virus released with the various cellular lesions as they develop. Presumably, this may best be done by simultaneous infections of all cells with a relatively high multiplicity of virus infectious units. Appropriate reference has been made in the text to the few cases where this has been done. More studies which correlate the fluorescent antibody technique with other methods are needed. Coffin and Liu (1957) were able by this technique to identify the specific intracellular localization of distemper virus and to separate this from other cellular lesions.

6. Tumor viruses have not been shown to differ from other viruses in their localization in the cell or in their intracellular lesions. Virus is found in the cytoplasm, in the microvilli, and within the nucleus in varying degrees. Its association with mitochondria is unsettled, possibly because most of the viral studies have concentrated on the obviously diseased cell, a factor which would unerringly select the cell in which virus and host were out of balance and virus was overcoming host cell. No significant morphological data on the balanced malignant cell produced by virus infection is available.

TABLE II

LOCATION OF VIRUS AND CELLULAR LESION IN SOME VIRUS TUMORS

Virus	Location in cell	Predominant lesion
Rous chicken sarcoma	Surface, occasional intracytoplasmic vesicle	Paranuclear hypertrophy
Mammary tumor of mice	Cytoplasmic vesicles, microvilli	Large cytoplasmic "inclusion" in some strains
Shope fibroma	Cytoplasmic	Cytoplasmic granular masses, nucleolar changes
Human warts	Nucleus, entire cell when keratinization takes place	Eosinophilic intranuclear inclusion
Frog adenocarcinoma	Cytoplasm, nucleus, microvilli	Intranuclear inclusion bodies in about 1/3 of tumors

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Chapter VI

Biological Aspects of Intracellular Stages of Virus Growth

ALICK ISAACS

National Institute for Medical Research, London, England

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I. INTRODUCTION

The information which we have at the moment of how animal viruses multiply within cells is insufficient to allow us to build up a connected picture

of the main processes involved. Present ideas have been largely shaped by work on bacteriophages, and, according to inclination, workers have tried to point to resemblances or differences between animal and bacterial viruses. At the moment, the most significant recent observations on virus multiplication are probably those of Hershey and Chase (1952) on bacteriophages and Gierer and Schramm (1956) and Fraenkel-Conrat (1956) on tobacco mosaic virus; these studies make it clear that infection can be initiated by virus nucleic acid preparations from which the bulk of the virus protein is absent. Recently Colter *et al.* (1957) and Wecker and Schäfer (1957a) have described similar experiments with animal viruses and the results suggest tentatively that infection could be produced by "nucleic acid extracts" of encephalomyocarditis (Mengo) and eastern equine encephalitis viruses (see also Section VII). Apart from these findings, our ideas on how animal viruses multiply are to a large extent governed by our interpretation of experiments on the eclipse phase and on virus recombination. Virus recombination is the theme of Chapter II; in this chapter the eclipse phase will be considered first, because of its importance to our concepts of virus multiplication.

Burnet (1955) defines viruses as microorganisms less than 0.4μ in diameter which can multiply only within living cells of a susceptible host, *and which undergo conversion into a noninfective form as a necessary step in their multiplication.* It is in this sense that the term "eclipse phase" will be used in this chapter. Burnet pointed out that in the great majority of animal virus types it was not possible, on the basis of evidence then available, to conclude that an eclipse phase was present or absent. In the following section the experimental evidence bearing on the existence of an eclipse phase for a number of different animal viruses is reviewed.

II. The Eclipse Phase

One of the striking findings about bacteriophage multiplication is that within a short time of the initiation of infection, no infective virus can be detected in disintegrated bacteria and this eclipse phase persists until halfway through the lag period.* Many similar investigations have been carried out with animal viruses, but while in all cases the amount of virus which could be recovered in the lag period represented only a fraction of the amount taken up by the cells, in only one case was the situation strictly analogous to that found with bacteriophages, i.e., western equine encephalitis virus (Rubin *et al.*, 1955) in which no virus was recovered from cells during the lag period. In all other cases some infective virus could be recovered throughout the lag period, ranging from an extremely small fraction to quite

* For the present purpose the term "lag period" is used to indicate the time between the initiation of infection and the appearance of newly formed virus.

a large proportion of the virus taken up. Since most studies with animal viruses have not been carried out with isolated cells, two interpretations of these results are possible. In the first, it is assumed that the virus which is detectable during the lag period is the parent of the new virus produced, and the virus which is not detectable has been destroyed by the cells and plays no further role in multiplication. In the second interpretation it is thought that the virus which is detectable during the lag period is adventitious, while the virus which has become undetectable has entered the eclipse phase on the road to producing new virus. It is also possible that the process is not uniform throughout animal viruses, although it would obviously be preferable to try to fit the experimental results into a single theoretical framework. We may therefore consider separately the following questions.

1. How much of the infectious virus taken up by the cells is recoverable during the lag period?

2. What happens to other viral properties, e.g., antigenic properties, during the lag period?

3. What is the significance of the infective virus which is detectable during the lag period? Thereafter, we may try to arrive at an assessment of the idea of an eclipse phase as a general phenomenon among animal viruses and as an essential stage in the multiplication process.

It is perhaps unfortunate that more work on the multiplication of animal viruses has been carried out on influenza and related viruses than on any others, but an attempt will be made to balance observations on influenza viruses with the findings in regard to smaller viruses, e.g., poliomyelitis and the encephalitis viruses and larger viruses, such as vaccinia and herpes simplex.

A. Amount of Infective Virus Recoverable during the Lag Period

1. *Bacteriophages*

As a standard with which to compare animal viruses, the experiments of Doermann (1952) on the eclipse phase of bacteriophages should be quoted. Doermann studied coliform bacteria infected with T4 phage. At intervals after infection the bacteria were lysed by applying a large dose of heterologous phage along with cyanide to stop further phage synthesis, a treatment which was shown to liberate as much phage during the terminal stages of intracellular development as occurs naturally. When bacteria were infected at a multiplicity of 1, lysis at $10\frac{1}{2}$ minutes after infection revealed less than 0.01 % of the final yield, or less than 1 phage particle in 80 bacteria. Clearly, therefore, the eclipse phase in its strictest sense implies that cells which are known to be infected with virus and which would have produced new virus if they had been left, show no infective virus during the lag period.

There are many difficulties in applying these techniques directly to the study of animal viruses, where, until recently, it has not been possible to infect uniform populations of isolated suspended cells. The advantages enjoyed by workers with bacteriophages are that the infectivity of their virus is usually stable at 37°C.; that adsorption of phages to their host cells is usually efficient; that the assay of infectivity is simple and has a relatively low error; that it is possible to assay without difficulty the number of infected cells; that the bacterial cells can be effectively isolated from one another to prevent spread of virus from cell to cell; and that in the cases which have been studied most there is a one-to-one ratio between infectivity and total virus particles present, as determined by electron microscopic counts (Luria *et al.*, 1951). In some or all of these respects workers with animal viruses have been at a great disadvantage. Most studies of this kind have been carried out with whole animals, tissues such as the chick chorioallantoic membrane, or populations of cells cultured *in vitro* under conditions where it is not known how many cells are supporting virus multiplication. In addition, many animal viruses are inactivated at 37°C. at a rate which appreciably affects the interpretation of the results of virus absorption studies, and often the absorption itself is not very efficient. In titrations carried out by methods other than pock- or plaque counting techniques the error of the titrations is usually high (Dulbecco, 1955); finally, with all animal viruses which have been adequately studied so far the minimal infective dose corresponds to at least 5 to 10 virus particles counted by electron microscopy. These difficulties should make for caution, and perhaps a little sympathy, in the interpretation of studies on the eclipse phase of animal viruses.

2. *Small Animal Viruses*

Rubin *et al.* (1955) infected a monolayer of chick embryo cells with a high multiplicity of western equine encephalitis virus. After allowing 30 minutes at 37°C. for virus adsorption, the monolayer was washed and trypsinized and the suspended cells washed and diluted greatly to prevent reinfection of cells. This was taken as zero time and the diluted cells were then incubated at 37°C. in buffer. At intervals, samples were removed and assayed for the total number of infected cells by direct plating on a cell monolayer. At the same time, the intracellular virus content was measured by plating out cells which had been washed to remove superficially adsorbed virus and disintegrated by ultrasonic vibration (a procedure which was shown to have no detectable effect on virus infectivity). The sample at time "zero" was found to contain 1.3×10^4 infected cells per milliliter, but 2.9 ml. of an aliquot, when disintegrated, revealed no intracellular infective virus. This corresponds to a recovery of less than 1 virus particle in about 40,000 infected cells.

Sanders (1953) found that when mice were inoculated in the tongue with the GD VII strain of encephalomyocarditis (EMC) virus, spread of virus occurred along the nerve to the hypoglossal nucleus. If this nucleus was removed 48 hours after inoculating the tongue, a suspension prepared from frozen and thawed nuclei contained no detectable infective virus, whereas suspensions prepared by incubating intact nuclei *in vitro* at 37°C. developed 3000 LD_{50} of virus per milligram of tissue. In these experiments it was not possible to say how much virus was initially present in the intact nuclei before incubation. Recently, Sanders *et al.* (1958) have studied the growth of EMC virus in agitated cultures of Krebs 2 mouse carcinoma cells under conditions where the cells remain separate, the virus being titrated by a plaque technique. At a multiplicity of infection of 0.1, about 95 % of the virus is adsorbed to the cells within 15 minutes. During the lag period the amount of virus recoverable by breaking up the cells with glass beads was 10^4 plaque-forming units per milliliter, whereas the number of infective centers, determined by direct plating of intact cells, was 10^6 per milliliter. Hence, the recovery of virus during the lag period was 1 % of the number of infective centers.

As far as has been investigated, therefore, small animal viruses behave like bacteriophages in showing a low recovery of infective viruses during the lag period.

3. *Medium-Sized Viruses*

Hoyle (1948) noted that during the lag period there was a low recovery of influenza virus from an infected chick chorioallantoic membrane. Henle (1949) carried out much more detailed experiments on similar lines; he found that after injecting various doses of influenza virus into the chick allantoic cavity about 30 % of the virus infectivity of the inoculum could be recovered in the allantoic fluid during the lag period. Henle interpreted this to mean that 70 % of the inoculum was taken up by the cells, but in view of later studies, which showed that the infectivity of influenza virus is reduced during incubation at 37°C. at an appreciable rate (Horsfall, 1954; Paucker and Henle, 1955a), conclusions based on the amount of the more stable virus hemagglutinin taken up by the cells are probably more reliable. Cairns and Edney (1952) reported that over a wide range of virus dosage about 50 % of influenza virus hemagglutinin was taken up by the allantoic cells during a 4½ hour incubation period. Hoyle and Frisch-Niggemeyer (1955) arrived at a very similar figure for virus labeled with P^{32} by studying the residual radioactivity in the allantoic fluid after allowing a 1½ hour period of absorption. Horsfall (1954), on the other hand, found an exponential decline in the titer of unadsorbed virus with time. When Henle's (1949) figures are calculated on the conservative basis that 50 % of the seed virus is taken up by the cells, it

appears that only 3.2 %, on the average, of the amount of infective virus taken up could be recovered from the membranes during the lag period. Although a number of different techniques of extraction were used, the recovery of virus was not improved.

Schäfer and Munk (1952) inoculated very large doses (10^9— 10^{10} LD_{50}) of fowl plague virus into de-embryonated eggs, washed out the inoculum with buffer 30–105 minutes afterwards, and then measured the infectivity of homogenized membranes. During the lag period, the infective titer was 10^{-5} to 10^{-6}. This represents 0.01 % of the virus inoculum, but it is not known how much of the inoculum was actually taken up by the cells.

Granoff (1955) inoculated $10^{7.4}$ LD_{50} of Newcastle disease virus (NDV) into the allantoic cavity of chick embryos and calculated that 80 % of the seed was adsorbed after 2 hours (this figure would be an overestimate if a significant degree of virus inactivation occurs during 2 hours' incubation at 37°C.). However, less than 1 % of the inoculated virus could be detected in ground membrane extracts at this time. More recently, Rubin *et al.* (1957) studied the growth of NDV in monolayers of chick embryo lung epithelium. The technique was similar to that used by Rubin *et al.* (1955) for western equine encephalitis virus. During the lag period, the number of virus infective doses recoverable by freezing and thawing the cells was about 1 % of the number of cell yielders (or infective centers). This proportion remained constant throughout the lag period.

Rubin (1955) studied the development of the Rous sarcoma virus in suspensions of tumor cells. By inoculating cells onto the chick chorioallantoic membrane, it was shown that the number of cells capable of initiating pocks was about 1/8 (range of 1/1.9 to 1/17 in different experiments) of the total number in the suspensions. These cells release virus at a slow rate during incubation at 37°C., but when the cells were frozen and thawed three times before incubation (a procedure which of itself did not reduce virus infectivity) the amount of virus released corresponded to one infective dose for 250 cells. On the average, therefore, the recovery of virus during the lag period was about 3 virus particles for every 100 infected cells.

The recoveries found for influenza and Rous sarcoma viruses during the lag period are slightly higher than the level of about 1 % (or less) of the virus taken up, which was found for the remaining viruses. However, at least for influenza viruses, this estimate is probably too high, as will be described in Section II, C.

4. *Larger Viruses*

A number of recent studies bear on the recovery of vaccinia and herpes simplex viruses from infected chorioallantoic membranes during the lag period, and, in general, the recoveries found have been higher than those

described for small and medium-sized viruses. Briody and Stannard (1951) and Crawford and Sanders (1952), working with vaccinia virus grown, respectively, on the chick chorion and in the rabbit skin *in vitro*, described a decrease in the number of infective particles that could be detected in the lag period, but the uptake of virus by the cells was not measured, so that the actual extent of the decrease is not known. Anderson (1954) inoculated eggs with 250 infective doses of vaccinia virus on the chorion and measured the amount of virus that could be recovered from ground membranes after various periods of incubation. The number of infective doses recoverable at 30 minutes was 70; at 1 hour, 350; this gradually declined to 18 infective doses at 9 hours, followed by a steep increase. This suggests that the virus was gradually adsorbed to the membrane over the first hour and that, as the virus entered the cells, there was a decrease in the amount of recoverable virus to about 7 % of that inoculated; from the titers obtained at 1 and 2 hours, it appears that most of the virus inoculated was taken up by the cells. Metcalf (1955) described a slightly greater decline in titer following inoculation of 7×10^4 infective doses, but in this experiment the uptake of virus was not measured. By contrast, others who have worked with vaccinia virus report much higher recoveries during the lag period. The interpretation of Maitland and Tobin's (1956) results is complicated, however, by what they call the "enhancement effect." They found that when a vaccinia elementary body suspension was prepared from rabbit skin by differential centrifugation and inoculated on the chorion, the amount of virus recoverable from the liquid on top of the membrane immediately after inoculation was considerably higher than the apparent titer of the inoculum (5 to 22 times in some experiments). This is presumably some sort of disaggregation effect; after this apparent rise in titer the virus entered the membrane. Thereafter the total virus concentration decreased, until at 2 to 4 hours it was 20 to 80 % of the peak titer (or 2 to 4 times the amount apparently inoculated) and most of this virus was recoverable from the membrane itself. Maitland and Magrath (1957) studied this decline in titer in more detail, using the same virus grown in chorioallantoic membrane *in vitro*. When pieces of chorioallantoic membrane were incubated with virus for 10 minutes, about 20 to 40 % of the inoculum became attached to the membrane. However, about three-quarters of this virus could be removed by washing, and the remainder constituted the baseline. Following incubation of washed pieces of membrane there was a slow decrease in the amount of virus recoverable. At 8 to 12 hours, the minimal titer was reached when about 20 to 50 % of the baseline value was recoverable.

A similar result was obtained with minced chick embryo cells. Trypsinized cells were left in contact with vaccinia virus for 10 minutes at 37°C., incubated *in vitro*, and titrated at intervals along with their suspending medium after disintegrating the cells mechanically. The base-line titration

showed 1 infective dose of virus for every 25 to 50 cells, and between 50 and 90 % of this amount was recoverable throughout the lag period. It seems important to repeat these experiments with seed virus which does not show the enhancement effect. Overman and Tamm (1957) incubated very large doses of virus (18×10^6 infective doses) with pieces of chorioallantoic membrane *in vitro*. The amount of virus taken up by the cells was not measured but there was only a slight decline, roughly 2-fold, between the third and eighth hours of incubation in the amount of virus recoverable from ground membrane pieces.

Shaffer and Enders (1939) noted that $1\frac{1}{2}$ hours after inoculating 120 infective doses of herpes simplex virus on the chick chorioallantoic membrane, only 10 doses could be recovered from ground membranes. However, further experiments with antiserum made it appear that only a small proportion of the virus had been taken up by the membrane in that time. Scott *et al.* (1953) carried out similar experiments but with a much larger inoculum, 8×10^4 infective doses. The recovery of virus from ground membranes after 1, 4, and 6 hours' incubation was usually less than 10 infective doses, but the uptake of virus during this time was not measured. In further experiments of the same kind, Modi and Tobin (1954) obtained a recovery of 1 to 10 % of the inoculum in the interval 1 to 6 hours after inoculation. In this case the inoculum was between 2.10^6 and 5.10^8 infective doses, but the amount absorbed is not known. Wildy (1954) carried out a series of most ingenious experiments to investigate the question of a possible eclipse phase for this virus. He inoculated eggs with 100 infective units of virus and at different time intervals eggs were rotated through 120°. The effect of this was that the bubble of air forming the artificial air sac was carried around to a new area of the membrane, taking along with it any virus which had not previously been fixed to the membrane. In this way it was found that the total amount of inoculated virus was partitioned between the two areas, 90 % of the inoculum being fixed to the first area in 90 minutes. The *total* amount of virus which could be recovered from membranes and overlying fluids declined gradually and was about 33 % of the original amount after 4 to 6 hours. In this experiment the inoculum was suspended in broth containing gelatin; after the period of incubation at 37°C., the eggs were chilled in order to solidify the gelatin and thus facilitate harvesting of all the virus in the overlying fluid. Since the gelatin was found experimentally to delay fixation of virus by the membrane, it is probable that part of the 33 % of the virus recovered between 4 and 6 hours represented virus not yet fixed to the membrane. Gostling and Bedson (1956) infected trypsinized chick embryo cell suspensions by contact with herpes virus overnight at 4°C. Under these conditions the uptake of virus was found to be only about 1 %, or less, of the inoculum. When such infected cell suspensions were incubated at 37°C., the level of infective virus recoverable from the cells

declined between the third and sixth hours to about 10 % of the level initially present. Gostling (1956) later showed that no further virus was recovered from such cells by extracting the nuclei with 6 % sodium chloride solution. Yoshino and Taniguchi (1956) have investigated in detail the question of an eclipse phase for herpes simplex virus. The virus was applied to a glass cover slip, which was then inverted onto the dropped chorioallantoic membrane. Under these conditions and over quite a large range in inoculum size, about 80 % of the inoculum was apparently absorbed by the cells within 30 minutes and 20 % remained unabsorbed for some hours. When extracts of chorioallantoic membrane were prepared, the residual unabsorbed virus became included with the virus present in the membrane, so it was necessary to carry out elaborate washing of the membrane to remove superficially absorbed virus. These experiments are discussed in Section C, which deals with the significance of the virus recovered during the lag period.

Recently Stoker and Ross (1958) studied the growth of herpes simplex virus in sheets of HeLa cells. Infection was initiated at high multiplicity of exposure and most of the extracellular virus was then removed by washing and treatment with antiserum. The proportion of intact infected cells was determined by titrating cell suspensions on the chick chorioallantoic membrane during the lag period; in different experiments it varied between 2 and 88 % of the total number of cells. After disintegration in distilled water in a microblendor, cell debris gave a significantly lower yield of infective virus than intact cells, the recovery measured in this way being about 10 %. (Neither the method of disintegration nor the cell debris reduced the infectivity of free virus.) Thus the amount of virus detected in the lag period corresponded to about one-tenth of the number of intact infected cells found to be present. Therefore, the majority of cells which would ultimately yield virus did not reveal any infective virus when disintegrated during the lag period.

The very large "viruses," or Chlamydozoaceae, have some interest in connection with the question of an eclipse phase, since they occupy a position intermediate between smaller viruses and rickettsiae. Girardi *et al.* (1952) inoculated chick embryos by the allantoic route with about 2×10^6 infective doses of meningopneumonitis virus and measured the content of virus in extracts of chorioallantoic membrane after different intervals of incubation. There was an increase in titer in the membranes as virus was taken up and this was followed by a slow and constant decline over a period of 20 hours. At that time the titer in the membranes was about 1 % of that initially present. Morgan (1956) has studied the growth of psittacosis virus in chick embryo mince which had been starved of essential metabolites. After inoculating $10^{3.5}$ infective doses (LD_{50}) of virus, the infectivity was rapidly lost and after 6 hours' incubation in starved cells titration of ground cells did not reveal any virus. In this case the recovery of virus was less than 0.03 % of that inoculated.

However, in spite of the absence of demonstrable virus, addition of beef embryo extract caused an immediate and rapid production of large amounts of virus.

A variation of these techniques occurs in the experiments of Girardi *et al.* (1952) on the recovery of meningopneumonitis virus during the lag period, and similar methods were used later by Sanders (1953), Anderson (1954), and Wildy (1954) in studies with encephalomyocarditis, vaccinia virus, and herpes viruses. The principle of the method is that a piece of tissue is removed during the lag period, part is homogenized and tested directly for its virus content, while an aliquot is cultured *in vitro* and then tested for its viral content. The object of the technique is to refine the experiments on recovery of virus during the lag period by removing a piece of tissue and stopping further virus absorption or spread of virus from neighboring cells or tissues. The method has given recoveries of virus in the homogenized tissue as compared with the cultured tissue of roughly 1 % for meningopneumonitis, 7 % for vaccinia (Anderson, 1954) and 10 % for herpes simplex (Wildy, 1954).

It is difficult to summarize these varied studies on the recovery of different viruses during the lag period. In many, there is not sufficient information to allow us to estimate what proportion of the virus absorbed by the cells became undetectable, but in those studies which allow us to estimate the recovery, it is clear that, as a general rule, a large proportion of the virus absorbed loses its infectivity shortly after entering the cells. Taking the recovery of less than 1 % found for bacteriophages as a standard, and making a few assumptions about the experimental data, it appears that losses in infectivity of about this order of magnitude, or slightly less, have been described for western equine encephalitis, encephalomyocarditis, influenza, fowl plague, Newcastle disease, Rous sarcoma, meningopneumonitis, and psittacosis viruses. For herpes simplex the recoveries reported by Gostling and Bedson and by Wildy may be about 10–20 %; for vaccinia virus, recoveries of between 7 and 90 % have been reported by different investigators. Leaving vaccinia and herpes aside temporarily, these large losses in virus infectivity might be thought of as due to breakdown of the virus after entering the cells or to the fact that the infectivity of animal viruses is often a very labile property which might be unable to withstand the coarse methods of extraction needed to prepare infected cell suspensions. If the latter explanation were correct, it might be possible to show the presence of intact virus during the lag period by testing for a more stable viral property, such as its antigenic behavior.

B. Attempts to Demonstrate Virus during the Lag Period by Its Antigenic Properties

Most attempts to detect virus during the lag period by its antigenic behavior have been carried out with the influenza virus hemagglutinin.

Hoyle (1948) described a typical experiment in which an egg was inoculated with 1024 hemagglutinating units of virus. After 3 hours' incubation, only about 10 % of the hemagglutinin remained in the allantoic fluid and presumably the rest had been taken up by the cells. However, when the membrane was ground with sand and incubated for 8 hours to allow elution of virus from cells, no hemagglutinin could be detected; this is less than 0.5 % of the amount that might have been expected to be present. Similar findings are recorded by Henle and Henle (1949), i.e., no hemagglutinins were recoverable from membranes in the first 2–3 hours after the use of very large inocula. But in this study no attempt was made to remove normal inhibitors of agglutination in the membrane; this factor could, in theory, influence the recovery of hemagglutinin from membrane extracts, depending on the dosage and strain of virus used. However, Isaacs and Edney (1950) showed that the use of RDE (receptor-destroying enzyme of *Vibrio cholerae*) to inactivate chorioallantoic membrane inhibitor did not increase the recovery of hemagglutinin. In their experiments, less than 1 % of the hemagglutinin taken up by the cells could be recovered in membranes which were ground and treated with RDE. They also tested membranes which had absorbed large doses of influenza virus for evidence of virus enzymatic (neuraminidase) activity during the lag period. Chorioallantoic membranes which had absorbed between 400 and 700 agglutinating doses of virus, each, were incubated for short periods at 37°C. in saline to allow elution of superficially absorbed virus. They were then ground and aliquots were incubated at 37 and 0°C. for 18 hours and the agglutinating inhibitory titer of the membranes measured. If any virus enzyme was present it should inactivate the inhibitor of agglutination during incubation at 37°C. but not at 0°C. In fact, no such enzymatic activity was demonstrated, whereas control membranes incubated with 100 agglutinating doses added after grinding had their agglutinating inhibitory titer reduced by more than 96 % under the same conditions. The fact that during the lag period neither the hemagglutinating nor enzymatic activities of the virus could be detected suggested that a fundamental change in the majority of the virus particles occurred after they entered the cells. Confirmation of this idea was obtained in similar experiments with heat-inactivated virus. Very large doses of heated virus (roughly 5000–10,000 agglutinating doses of virus per egg) were absorbed by chorioallantoic membranes, which were then ground and treated with RDE. Such extracts showed no evidence of viral hemagglutinin, or of antigenic activity as determined *in vitro* by antibody-combining activity, or *in vivo* by antigenicity in mice. By contrast, heated virus incubated with chorioallantoic membrane extract *in vitro*, and then treated with RDE, showed high activity by all three tests.

Tamm and Tyrrell (1954) studied the recovery of influenza virus hemagglutinin after 1 hour's incubation with pieces of chorioallantoic membrane

in vitro. After homogenizing, the membranes were treated with RDE and 3.6–9.4 % of the virus which had been absorbed was recovered. Hoyle and Frisch-Niggemeyer (1955) studied the radioactivity and hemagglutinin content of chorioallantoic membranes which had absorbed 480 agglutinating doses of P^{32} labeled influenza virus during a period of 1–2 hours' incubation. In extracts of the membrane, prepared by freezing and thawing and treatment with RDE, there was no demonstrable hemagglutinin, although a titer of 140 would have been expected on the basis of the radioactivity. The fact that the radioactivity was not due to hemagglutinin masked by combination with membrane inhibitor was shown by centrifuging the extract at 100,000*g* for 3 hours, when the greater part of the radioactivity remained in the supernatant. Hoyle and Frisch-Niggemeyer also observed that a high proportion of the phosphorus label could be found in the "residual membrane," i.e., the sedimentable brei from the original membrane extract. This fraction was not apparently tested for its viral content, but Henle (1949) had shown earlier that when extracts of infected chorioallantoic membrane were prepared during the lag period and centrifuged lightly (2000 r.p.m. for 20 minutes) the bulk of the virus infectivity remained in the supernatant. Isaacs and Lindenmann (1957) found that when heated influenza virus was absorbed by chorioallantoic membranes *in vitro* and then the membranes were washed, the interfering activity of the virus could not be detected in extracts of the membrane before incubation. If such membranes were incubated for 3 to 6 hours at 37°C. before extraction, interfering activity was found in the membranes and was later secreted into the surrounding fluid, but this interfering activity was not due to the heated virus but to a product of the cell-virus interaction, which they called interferon.

These examples show that during the lag period of influenza virus growth there is a low recovery of hemagglutinin and of virus enzymatic activity which closely parallels the low recovery of infective virus. The finding with heated influenza virus that hemagglutinin, viral antigenic activity, and interfering activity were lost suggests a fundamental alteration in the viral particle on entering the cell; this point is discussed later. It is unfortunate that only influenza viruses have been studied for their antigenic behavior during the lag period, but the problem is technically more difficult with other viruses. So far as they go, these results add emphasis to the low recoveries of infective virus during the lag period.

C. The Significance of Virus Recoverable during the Lag Period

In most studies described above small amounts of virus could be extracted from infected cells during the lag period, and we can now consider the significance of this recoverable virus. The most important consideration in relation to the question of an eclipse phase is whether the recoverable virus

is situated in an extracellular or an intracellular position at the time the cell extract is prepared for virus assay. The experimental method most commonly used to answer this question has been to find the effect of treating the cells with viral antiserum on the amount of virus recoverable after disrupting the cells. There is, however, a possible objection to this technique, that antibody might first combine with extracellularly situated virus, and, if bivalent, later combine also with intracellular virus when the cells are disrupted. Efficient removal of the antibody by washing is therefore an essential part of the technique, and if it is shown that treating control cells with antiserum after infection has been initiated does not reduce the final yield of virus from these cells, this can be taken as evidence that the antibody has not had much influence on the titer of intracellular virus. Two other types of experiment have also been carried out to test the localization of recoverable virus, i.e., treating the cells with RDE in the case of influenzal infection, and repeated washing of cells to see what is the effect on the amount of virus recoverable during the lag period.

Andrewes (1930) first showed that the growth of herpes simplex virus in tissue culture was not inhibited by viral antiserum inoculated after the initiation of infection. The effect of antiserum used in this way on the recovery of virus during the lag period has been studied by a number of workers. Henle and Henle (1949) noted that antibody given 30 minutes after a large inoculum of influenza virus ($10^9 LD_{50}$) in the chick allantoic cavity reduced the recovery of virus in the membranes during the lag period by 3 $\log_{10}$, without reducing the eventual yield of infective virus in control eggs similarly inoculated. Apparently, too, antibody was not fixed by uninoculated membranes. On the other hand, antibody reduced the yield of virus after a small inoculum, suggesting that some multivalent antibody combined with superficially absorbed virus and was then carried over into the tissue on extraction. It is best, therefore, as Henle and Henle point out, to accept the findings as giving qualitative evidence that a substantial part of the recoverable virus is superficially attached to the cells, without attempting to interpret the findings on a strictly quantitative basis. In essentially similar experiments, Schäfer and Munk (1952) found that the recovery of fowl plague virus from the membrane was reduced by 3–4 $\log_{10}$ by treatment with antiserum; Tamm and Tyrrell (1954) and Ackermann and associates (1955) found a 1 $\log_{10}$ reduction with influenza virus grown in pieces of chorioallantoic membrane *in vitro*; and Ishida and Ackermann (1956), while investigating the effect of temperature on the fixation of virus to cells, noted that immune serum treatment caused a large reduction in the recovery of influenza virus after adsorption to membranes at 3°C. However, the latter workers also obtained, in the same way as Henle and Henle (1949) had done, results which suggested that in their experiments some serum becomes bound to the

cells in association with superficial virus and may later mix with intracellular virus on grinding the cells. Gostling and Bedson (1956) found that chick embryo cell suspensions incubated with herpes simplex virus overnight at 4°C. and then treated with diluted antiserum overnight at 4°C. contained throughout the lag period about one-tenth the amount of virus of control cells in which the antiserum treatment was omitted. In these experiments evidence was shown that there was insufficient antiserum present to affect the results of the infectivity titrations and the findings are attributed to neutralization of superficially adsorbed extracellular virus. As mentioned earlier, the level of virus extractable from the cells then declined to one-tenth of the initial level, but this fraction of the initially absorbed virus was not neutralized by treatment with antiserum in their experiments. In the experiments of Yoshino and Taniguchi (1956) an antiserum in a 1/100 dilution was used to treat chorioallantoic membranes one-half hour after inoculating herpes simplex virus by the cover slip technique mentioned above. The use of antiserum was combined with an elaborate wash-drying procedure involving numerous washings for each membrane in buffered saline with careful drying on filter paper between washes. With this method about 0.0015 to 0.004 % of the inoculum was recoverable during the lag period compared with about a 20 % recovery in membranes not treated in this way. These workers also attempted to remove superficially adsorbed virus by experiments involving washing the membrane *in situ*, followed by sponging with gauze after each wash. After 5 washings with sponging between each, the amount of recoverable virus was about 1 % of that present before washing; this degree of washing apparently did not harm the cells too much, as judged by the yield of virus from similar membranes incubated at 35°C. However, with 6 washings, involving sponging with 3 changes of gauze between each washing, the yield of virus was drastically reduced following incubation at 35°C. Such a traumatic procedure might easily damage cells so that intracellular virus could be washed away. These experiments display novel methods of trying to answer this problem and it would be interesting to see them applied to vaccinia virus.

That tissues which had adsorbed influenza virus when repeatedly washed (15 times) in buffer continued to release virus into the washings was demonstrated by Ackermann *et al.* (1955), and similar findings were noted after 12 washings of cells infected with herpes simplex virus (Gostling and Bedson, 1956). One method used to remove superficially adsorbed influenza virus was to treat the intact chorioallantoic membranes with large doses of RDE, followed by thorough washing before grinding (Isaacs and Edney, 1950). With the use of RDE the recovery of infective virus was reduced 10-fold, i.e., 0.04 % of the seed compared with 0.6 % in membranes which had not been washed with RDE. Insufficient RDE was present to interfere with the assay of infectivity.

These experiments making use of antiserum, repeated washing, and RDE have not always given unequivocal answers but, in general, it seems that in nearly all the cases investigated most of the virus recoverable from cells during the lag period is extracellular and can be removed by one or other of these methods. Nevertheless, it has always been found too that a very small fraction of the virus taken up by the cells can be detected in the lag period and is not completely removed by antiserum, washing, or RDE. This virus is therefore assumed to be intracellular, and, depending on our point of view, we may regard it (in experiments in which isolated cell suspensions were not used) as the parent of the new virus yield which will remain infective throughout the lag period, or as virus which is about to enter into an eclipse period, or as adventitious virus of no significance in the multiplication cycle. Quantitative considerations, however, make the first explanation untenable for many of the above viruses. Thus, if we assume as average figures for influenza and fowl plague viruses that 0.1 % of the virus taken up can be detected as intracellular virus during the lag period, we are obliged to conclude that only 1 out of every 1000 infective virus particles taken up by the cell survives to multiply, while the remaining 999 perish. But there is good evidence from electron microscopic counts and indirect counting methods that infection with these viruses can be initiated by 10 virus particles (Isaacs, 1957). When we consider, too, the results of Hoyle and Frisch-Niggemeyer (1955) on labeled influenza virus, it seems justifiable to conclude that on entering the cells the majority of influenza virus particles become changed into material which is either smaller or less dense than the original virus and which lacks infectivity and many of the other properties of the virus; and that this change may well be analogous to the eclipse phase of bacteriophages and cannot be explained other than as a stage in the life cycle of the virus.

At the moment we cannot say what is the significance of virus which is not neutralizable by serum but is recovered during the lag period.

With other viruses, the recovery during the lag period is almost as low as that found for bacteriophages, but one cannot yet be certain that this is a true eclipse. The findings with herpes simplex virus are conflicting. Wildy (1954), Yoshino and Taniguchi (1956), and Stoker and Ross (1958) favor the idea of an eclipse phase while Gostling and Bedson (1956) think it is not proved; the same conclusion was reached by Maitland and co-workers for vaccinia virus. Indeed, Maitland and Magrath (1957) report that a decline in infectivity of vaccinia virus paralleling that which occurred in experiments with intact chorioallantoic membrane was found when the virus was adsorbed to membranes which had been heated at 56°C. for 20 minutes, a procedure which destroyed the ability of the membranes to support virus multiplication. The evidence cited for vaccinia and herpes simplex viruses seems therefore to be insufficient at the moment to decide definitely in favor of or against an

eclipse phase, although the findings of Stoker and Ross (1958) are in favor of an eclipse phase for herpes virus. More decisive evidence, particularly for vaccinia virus, will be provided by experiments along the lines of Rubin *et al.* (1955) with isolated cells, or if recovery of virus in the lag period is found to be low compared with the "plating efficiency," i.e., the ratio of infective virus count to total virus particle count.

Nevertheless, the trend of the findings favors the idea of the eclipse phase as a general phenomenon among animal viruses. Morgan's (1956) experiments with psittacosis virus are very difficult to interpret except on the assumption that the virus is in a noninfective phase in the starved cell and that it resumes its development when the cell is supplied with certain essential metabolites. The studies by Dulbecco and Vogt (1955) and Dulbecco (1957) on the sensitivity of poliomyelitis virus in infected cells to ultraviolet irradiation (based on the work of Luria and Latarjet (1947) on bacteriophages) also imply a change of state of the virus on entering the cells; from the evidence on influenza viruses, a change of state to a noninfective phase seems to be the fate of the majority of infecting virus particles.

III. Development within Infected Cells of Antigens Associated with Virus Multiplication

The development of fully mature virus in infected cells may be preceded or accompanied by the development of associated viral antigens. These antigens have excited interest and speculation as to the possibility that some of them might be building blocks which are later assembled to make mature virus. Unfortunately, although there are many published studies of the development of associated viral antigens, attempts to inculpate them as viral precursors have been carried out mainly with influenza and related viruses, with which, therefore, this section is mainly concerned.

A. The 30 S Complement-Fixing (Soluble) Antigen of Influenza and Related Viruses

During the growth of influenza and related viruses there develops in infected cells an antigen detectable by the complement fixation test, of much smaller particle size than the mature virus particle (Hoyle and Fairbrother, 1937). This antigen has a sedimentation constant of 30 S, compared with about 700 S for the virus particle, and is often called the influenza soluble antigen or S, as opposed to the viral antigen or V. The viral antigen has a greater serological specificity than the soluble antigen, and influenza A viruses with widely divergent antigenic characters are said to have a common soluble antigen. However, observations on complement fixation with the

soluble antigen have usually been made with sera from adults convalescent from influenza, although such sera are unsuitable for tests with viral antigen on account of their broad reactivity with different strains of influenza A virus. In order to measure specific viral antigens most workers use sera of convalescent or immunized animals which have not had previous experience with influenza virus and therefore respond to infection or immunization in a more specific way. When sera from infants infected (presumably for the first time) with influenza virus were tested with soluble antigens prepared from different influenza A strains, the soluble antigens showed some strain specificity (Grist, 1957). There is therefore a real difficulty in defining the influenza soluble antigen precisely. It is normally taken to mean an antigen of sedimentation constant 30 S, present in infected tissues, and showing the broad serological reactivity of the virus serotype. It must be emphasized however, that the term soluble antigen may have been used by different workers to describe different things; this is especially true when studies with human convalescent serum are compared with investigations using animal sera reacting with soluble antigen.

Hoyle (1948) found that in cells infected with influenza virus, soluble antigen could be detected at the end of the eclipse period and before the formation of mature virus. Subsequently, Hoyle (1950) showed that, on treating virus elementary bodies with ether, soluble antigen was liberated, and he therefore suggested that the soluble antigen represented the essential form in which the virus multiplied within the cell. This is an attractive theory which attempts to draw an analogy between the influenza soluble antigen and the nucleic acid of bacteriophages and tobacco mosaic virus. At the moment, the evidence concerning this theory is as follows:

1. Soluble antigen is detectable in tissues within 3 hours of inoculating influenza virus, whereas fully infective virus is not formed until the fourth to the fifth hour (Henle and Henle, 1949). However, while this finding has been generally confirmed, there is one reservation which should be made. In experiments on the time of appearance of soluble antigen, large virus inocula are required to produce measurable yields of soluble antigen, and under these conditions multiple infection of cells occurs and incomplete virus is formed (see Section VI). Hence, to overcome this objection, the rise of viral infectivity must be studied after the use of small inocula; this means that the development of soluble antigen and infective virus have not been observed under comparable conditions. There are a number of known instances with other viruses in which newly formed virus has been shown to appear earlier with large inocula, e.g., Dulbecco and Vogt (1954), so it is possible that the earlier appearance of the influenza soluble antigen may be more apparent than real. Recently, Ledinko and associates (1957) have described a rise in infectivity $2\frac{1}{2}$ to $2\frac{3}{4}$ hours after infection of chick embryo lung cells with

influenza virus when large doses of RDE were present in the medium. Hemagglutinin appeared at the same time but soluble antigen was not studied in this system. Hoyle first thought that the increase in soluble antigen in the cells occurred at a logarithmic rate but later (Hoyle, 1953) he agreed that a linear increase fitted the experimental observations better.

2. Hoyle (1950) interpreted his findings on the effect of ether treatment of influenza virus particles as showing that when the particles were disrupted they liberated soluble antigen which had been enclosed within the intact particle. Fulton (1953) interpreted the same findings as indicating that ether treatment of the virus had degraded the viral antigens and blunted their serological specificity in an analogous way to the effect shown by ether treatment of rickettsiae (Fulton and Begg, 1946). These differences in interpretation emphasize the difficulty of identifying soluble antigen derived from infected tissues with the antigen liberated by ether treatment of virus elementary bodies. In favor of Hoyle's interpretation are the findings of Lief and Henle (1956b) that with a standardized technique of ether extraction, a constant amount of soluble antigen was liberated from a given dose of influenza virus; in addition, less soluble antigen was liberated from incomplete virus than from standard virus. This finding is further discussed in Section VI, A, but the implication is that the amount of soluble antigen which can be liberated is closely bound up with the infectivity of the virus, a finding which would not be expected on Fulton's hypothesis.

3. Evidence has been produced that the soluble antigen is nucleoprotein or is closely bound to nucleoprotein. Hoyle (1952) showed that the serological activity of the soluble antigen was greatly reduced by treatment with trypsin, but only slightly reduced by prolonged incubation with ribonuclease, although the ribonuclease could have been contaminated with small amounts of protease. Hoyle also found that the soluble antigen was precipitated by lanthanum acetate.

More convincing evidence for the presence of ribonucleic acid in the influenza soluble antigen was provided by Ada and Perry (1954). They prepared extracts by differential centrifugation of infected chick embryo lungs from which the soluble antigen was precipitated by addition of immune mouse antiserum. Such precipitates were fractionated by a modified Schmidt-Thannhauser method and were found to contain roughly 4 % of RNA and 0.5 % DNA. Later, Ada (1957) prepared influenza soluble antigen from infected chorioallantoic membranes and compared it with virus soluble antigens prepared by ether extraction of the virus. The two antigens were found to have comparable complement-fixing activities per milligram of dry weight, but while both showed the presence of ribonucleic acid, the total amount of acid and the proportions of nucleotides differed greatly in the two preparations. On the other hand, Schäfer (1957) found that fowl plague virus

soluble antigens, prepared in the same way from infected tissues and from ether extracts of the virus, were very similar to one another in their behavior with antisera, nucleic acid content, and ultraviolet absorption spectrum.

4. Wiener and associates (1946) showed that soluble antigen could be liberated from influenza virus by ultrasonic vibration and this was confirmed by Lief and Henle (1956a). However, Lief and Henle found that after the ultrasonic treatment there was always more antigen which could be extracted subsequently with ether, and they interpreted this to mean that some soluble antigen was loosely adsorbed to the virus surface and some was present in the virus particles themselves.

In summary, while the evidence is incomplete, it seems likely that the soluble antigen is a nucleoprotein which is produced in infected cells before mature virus appears, and that some of it becomes incorporated in the virus particles. While this statement may be an oversimplification of the events which occur and leaves many points unexplained, it suggests further useful lines of investigation.

B. The Hemagglutinin of Myxoviruses

In parallel with studies of the appearance of influenza soluble antigen in infected cells, the development of viral hemagglutinin (or V antigen) has been intensively investigated. However, hemagglutination may be caused by the virus elementary bodies, by particles which are either smaller or less dense than the elementary bodies (see below), or by small fragments produced, e.g. by ether treatment of elementary bodies—all these particles showing similar serological specificity. Unfortunately, in most studies the particle size of the hemagglutinin is not mentioned. This means that a great deal of useful information on the development of viral hemagglutinin in infected cells cannot yet be applied to the problem of which antigens might qualify as possible viral precursors.

At this point it is necessary to anticipate some of the discussion in Section VI by considering the concept of incomplete viruses. Von Magnus (1946) showed that on serial passage of large inocula of influenza virus in the allantoic cavity incomplete virus was liberated into the allantoic fluid. The incomplete virus showed normal hemagglutinating and serological activity, but had a very low infectivity relative to that of virus similarly passaged at high dilution. There is still controversy as to whether incomplete virus represents virus which was prematurely liberated from the cells before it had acquired full infectivity, i.e., its normal development was interrupted; or whether it should be considered as malformed virus produced by overloaded cells. In the present discussion the term incomplete virus will be used strictly for the virus *spontaneously liberated from cells* when the experimental technique of von Magnus is applied. This limitation is necessary because there are two

other types of condition under which hemagglutinin of low relative infectivity is produced, but in both cases the hemagglutinin remains intracellular and is not normally liberated from the cells. The noninfective hemagglutinin produced in these other conditions shows some significant differences from the incomplete virus of von Magnus and therefore will not be called incomplete virus here. The first example was described by Granoff and associates (1950). They prepared extracts of chorioallantoic membranes infected with NDV and found that after high-speed centrifugation (20,000 r.p.m. for 20 minutes) the supernatant showed serologically specific hemagglutinin of low relative infectivity. Virus from the allantoic fluid did not behave in this way after high-speed centrifugation. Granoff (1955) later confirmed these results with NDV and found that the same phenomenon could be demonstrated with the PR8 strain of influenza virus A. He called this hemagglutinin "S" for small, although of course its size was not defined by the centrifugation results, and it might be merely less dense than the elementary bodies. By passage experiments it was shown that the "S" influenzal hemagglutinin did not reproduce incomplete virus on passage, thus differentiating it from the incomplete virus prepared by the technique of von Magnus. Schäfer and Munk (1952) have also reported finding hemagglutinin of low relative infectivity in the supernatant after high-speed centrifugation of membranes infected with fowl plague virus, and Schäfer (1957) has shown the appearance of this hemagglutinin on electron microscopy (Schäfer calls these incomplete forms). This membrane hemagglutinin consists of balloon-like particles of variable size measuring 50–550 mμ in diameter in the flattened state. It seems, therefore, that in NDV, fowl plague, and influenza infection there develops in infected membranes hemagglutinin of low relative infectivity, sedimenting less readily than the virus elementary bodies; this hemagglutinin is not normally secreted into the allantoic fluid. By contrast, the same membrane extracts also contain hemagglutinin which shows the infectivity and sedimentation behavior characteristic of mature elementary bodies; and this is normally secreted into the allantoic fluid. It seems possible that the so-called "S" hemagglutinin is one of the virus building blocks; further studies of its appearance in the tissues relative to that of mature elementary bodies might help to decide this point.

The second situation in which hemagglutinin of low relative infectivity is produced in cells is when there is a partial cycle of influenza virus multiplication, as was found in the mouse brain (Schlesinger, 1950), the chick chorion (Fulton and Isaacs, 1953), and HeLa cells (Henle *et al.*, 1955). In these three sites, infection by strains of influenza virus which have not been specially adapted to these particular cells results in the formation of soluble antigen and hemagglutinin in readily detectable amounts, but fully infective virus is formed to only an insignificant degree. In these three sites, too, the

hemagglutinin remains intracellular, as in the case of the "S" hemagglutinin. Also, the morphological appearances described by Werner and Schlesinger (1954) for influenza hemagglutinin from mouse brain are very reminiscent of the tissue forms described by Schäfer for fowl plague; in unpublished observations made in collaboration with R. C. Valentine, it was noted that viral hemagglutinin from infected HeLa cells showed the same morphological appearance as the forms described by Schäfer (1957). On the other hand, incomplete virus obtained by the method of von Magnus and present in allantoic fluid appears on electron microscopy (Donald and Isaacs, 1954c; Pye *et al.*, 1956) to be much less pleomorphic than the tissue form of the hemagglutinin, and von Magnus (1954) states that incomplete and standard virus do not show any pronounced differences in size and shape on electron microscopy. It is tempting therefore, to suggest that in the cells of these three sites there is a lack of some factor required for completing the virus multiplication cycle and that viral building blocks accumulate in the cells. There is, however, no firm experimental evidence which would let us identify any viral-associated antigen as a virus precursor.

Henle *et al.* (1956) studied the ratio of infectivity : hemagglutinin titer (I/HA ratio) in the membranes and fluid media of de-embryonated eggs infected with influenza virus. With seeds of different varieties, i.e., diluted or undiluted standard seeds and seeds prepared by serial passage of large inocula to produce incomplete virus (see Section VI), it was regularly found that the I/HA ratio in the membranes was about 1.5 log less than that of the liberated progeny. In any 2-hour liberation period about ten times the amount of infective virus was shed into the medium as was present in the membrane, whereas only about one-quarter of the hemagglutinin was released in the same time. In addition, V or viral antigen, measured by complement fixation and distinct from the viral hemagglutinin, could be shown to be present in the tissues but not in the fluid in any 2-hour period. Henle *et al.* also found that on adding potassium cyanide the infectivity and hemagglutinin titers in the membranes decreased sharply and rose again, together, as the cyanide was removed. They concluded that the hemagglutinin in the membrane was noninfective at first and acquired infectivity later in its development, the production of noninfective hemagglutinin and conversion to mature virus forming a dynamic process. The noninfective hemagglutinin in these experiments may be the same as the "S" hemagglutinin of Granoff *et al.* and it seems important to investigate this possibility.

In many studies, the times of first appearance of hemagglutinin, soluble antigen, and infective virus have been compared. As discussed earlier, it is difficult to compare strictly the time of appearance of mature virus with that of the other two antigens since they are not normally measured under identical conditions, although it appears that infective virus develops after

the other two antigens (Henle *et al.*, 1954). As between soluble antigen and hemagglutinin, Hoyle (1948) found that soluble antigen appeared first, while Fulton (1949) found that the two appeared simultaneously. It is important in making this comparison to be sure that the inhibitor of viral hemagglutination present in susceptible cells is completely inactivated; when this precaution was taken there was no difference between the time at which hemagglutinins and soluble antigen could be first detected (Liu and Henle, 1951; Burnet and Lind, 1954). It seems that the evidence that the soluble antigen appears before the hemagglutinin in infected cells is not definite.

C. Cell-Associated Antigens of Other Viruses

In almost all viruses which have been investigated, small particle antigens, detectable by complement fixation and usually called soluble antigens, have been found associated with viral multiplication. It has generally been found that these antigens remain intracellular, that they are distinctly smaller than the virus elementary bodies, and that they have less serological specificity than the elementary bodies.

There are at least two distinct cell-associated antigens of vaccinia virus, the soluble LS antigen (Craigie, 1932), which is a protein, and the hemagglutinin (Nagler, 1942), a lipid-protein complex. During the growth of the virus these antigens increase in amount parallel to the increase of viral infectivity (Metcalf, 1955; Maitland and Tobin, 1956) and there is no evidence of their appearing before mature virus or of their being used up to produce new virus. A soluble antigen is produced during the growth of herpes simplex virus (Hayward, 1949) but there is no evidence for its preceding the appearance of infective virus (Scott *et al.*, 1953; Modi and Tobin, 1954). Van den Ende and co-workers (1957) studied the soluble (12 mμ) antigen which develops in the brains of suckling mice infected with rabies virus. The antigen appeared after the rise in titer of infective virus and apparently continued to increase in amount after the maximal infective titer was reached. Differences in sensitivity of infectivity and complement fixation titrations affect the interpretation of all these results but there is no support for the idea that soluble antigens in general appear in cells before mature virus.

Among the smaller viruses, poliomyelitis (Selzer and Polson, 1954), African horse sickness (Polson and Madsen, 1954), and foot-and-mouth disease (Bradish *et al.*, 1952) viruses have all been shown to produce soluble complement-fixing antigens of small particle size; Polson has drawn attention to the fact that the estimated particle size for the soluble antigens of many different viruses is about 12 mμ. Apart from this, there is no evidence to suggest whether the soluble antigens of different viruses have a similar function or are produced by analogous processes. There is, however, an interesting

hint derived from studies of virus-infected cells stained with fluorescin-labeled antibody. Liu (1955) showed that fluorescence was first detectable in the nuclei of infected cells and that this nuclear fluorescence was caused by the presence of the soluble antigen; a similar observation has recently been made for fowl plague virus by Franklin (1957). Also, early nuclear fluorescence was found for herpes simplex virus (Lebrun, 1956), but it is not known whether the early fluorescence is due to elementary bodies or to the soluble antigen. If this point were investigated for a number of viruses it might give an important hint about the development and function of soluble antigens (see also Section VII).

IV. Dynamics of the Development of Infective Virus

In this section an attempt will be made to summarize studies on dynamic aspects of the development of fully infective virus in infected cells. Ideally such studies are carried out along the lines of the "one-step growth curves" of bacteriophages, i.e., an attempt is made to limit experimentally the growth of virus to a single cycle by preventing spread of the newly produced virus to neighboring cells. In practice, studies with many viruses have not yet reached this ideal. Nevertheless, an attempt will be made to analyze for a number of animal viruses the rate at which infective virus appears, the yield of virus (measured as infective doses) per cell and the duration of the growth cycle.

A. Poliomyelitis Virus

Dulbecco and Vogt (1955) carried out one-step growth curves of poliomyelitis virus of all three types in suspensions of isolated monkey kidney cells. The technique was the same as that which they used with western equine encephalitis virus (see Section IV, B). The cells are incubated with virus in buffer to allow adsorption of virus. The cells are then washed and diluted greatly in nutrient medium so as to minimize readsorption of released virus. They found that after a lag period of about 4 hours there was a sharp rise in the titer of extracellular infective virus, which increased for 2–3 hours and then tailed off. During the period of rapid rise in infectivity the increase occurred at a nearly exponential rate. The yield of virus was roughly 100 infective doses per infected cell for all three types. Lwoff *et al.* (1955) studied the kinetics of virus release from single cells. They used type 1 poliomyelitis virus grown in monkey kidney cells and deposited in drops of paraffin oil after they were infected. The lag period was $5\frac{1}{2}$–7 hours, a longer period than found for suspended cells cultures, perhaps because of the difficulties of the technique of culturing individual cells. Once virus liberation started, the virus was released extremely rapidly and 100–200 infective doses per cell were liberated

from the cells within about half an hour. Again the rapid liberation may have been a reflection of the culture conditions.

Howes and Melnick (1957) have also studied the growth of type 1 poliovirus in monkey kidney cell monolayers and they ensured that a single cycle of growth was observed by infecting their cells at a multiplicity of 4 or more. They found that after a lag period of 3 hours there was a rapid and, at first, exponential increase in the amount of cell-associated virus with the production of approximately 100 plaque-forming units of virus per infected cell. At about $5\frac{1}{2}$ hours, after the initiation of infection, 50 % of the total virus yield had been produced. The yield of extracellular virus lagged behind the cell-associated virus by about 1 hour.

B. Western Equine Encephalitis Virus

Dulbecco and Vogt (1954) used a dilution technique to make one-step growth curves of western equine encephalitis virus grown in chick embryo cell suspensions. The lag period was about $2\frac{1}{2}$ hours with a multiplicity of 4, and $3\frac{1}{2}$ hours with a multiplicity of 0.15. Once virus release commenced there was an exponential rise in the titer of extracellular virus for about $1\frac{1}{2}$ hours, followed by a slowing, with the maximal yield at 6–8 hours. The yield of virus was approximately 100–200 infective particles per cell, but conditions of cultivation were probably not optimal, since a yield of 200–1000 infective particles per cell was found when the virus was grown in a cell monolayer. With a high multiplicity of infection the yield of virus per cell was consistently higher than at low multiplicity, suggesting that more than one virus particle per cell can take part in the growth process. This last finding is an extremely interesting one, and should be tested in other virus systems with simultaneous study of the intracellular (or cell-associated) virus and the extracellular virus.

Rubin *et al.* (1955) studied the growth of this virus in a similar system but with particular attention to the rate at which intracellular or cell-associated virus developed. Monolayers were infected at high multiplicity and after a 30-minute absorption period the cells were washed and trypsinized and the suspended cells diluted greatly. After a lag period of about $1\frac{1}{2}$ hours the cell-associated virus, measured after rupturing the cells by ultrasonic vibration, was found to increase exponentially for a period of nearly 3 hours, the virus doubling in amount every 15 minutes. As described earlier, no virus could be detected in these cells at zero time, i.e., the time at which the cell dilution was made. Rubin *et al.* calculated that the average maximum number of cell-associated infective virus particles per cell at the end of the period of exponential rise was only 4 to 10, although each cell had spontaneously released 100 infective particles, i.e., the virus is very rapidly released when formed (this

point is further discussed in Section V). This suggests that the exponential rise in the amount of intracellular infective virus reflects a logarithmic rate of increase of one virus precursor, whose rate of production limits the rate of production of mature infective virus forms.

C. Influenza Virus

Henle *et al.* (1947) carried out one-step growth curves of influenza A and B viruses in the allantoic cavity of the chick embryo, using a large dose of ultraviolet irradiated heterologous virus as an interfering agent, to prevent the occurrence of a second cycle of virus growth. The validity of this technique depends on whether the irradiated virus can be used to prevent a second cycle of virus growth without inhibiting the first cycle. In their paper, Henle *et al.* (1947) thought that the irradiated virus interrupted the readsorption of virus released in the first cycle, but, since the irradiated virus was given 1 hour after the live virus, it may have also induced some interference in the cells initially infected. Hence the appearance of a step in the growth curve described by Henle *et al.* might be to some extent artificial, and indeed, in later work, Henle and associates (1954) showed that virus continued to be released from infected cells over a period of 30 hours or more. With this limitation, the results of Henle and his co-workers show that for the PR8 strain of influenza A, after a lag period of 6 hours, new virus was released into the allantoic cavity within the next 2 hours with an average value of 63 ID_{50} produced per ID_{50} of virus adsorbed. The corresponding figures for the Lee strain of influenza B were 36 ID_{50} produced per ID_{50} adsorbed after a lag period of 9 hours. Henle and Rosenberg (1949) later extended these findings to other strains of influenza A and B and found that, in general, the influenza B viruses showed a longer lag period and a lower viral yield than the A strains. However, if in fact the viral interference is induced at a constant time after inoculating the irradiated virus, the apparently lower yield may simply reflect a slower rate of growth of the B viruses and it is known that the yield of some influenza B strains per egg is not significantly less than for A viruses.

Cairns (1952) studied the release of influenza A virus hemagglutinin into the allantoic cavity and used the *V. cholerae* enzyme, RDE, to prevent readsorption of newly released virus. Viral hemagglutinin was first liberated after 5 hours but the 50 % liberation time, i.e., the time at which half the ultimate first-cycle yield of virus had been liberated, appeared to be about 8 hours. Cairns also measured the amount of virus liberated in each half-hour period ("differential response") in this system and found that periods of peak liberation of virus occurred at $7\frac{1}{2}$–9 hours, 14–$14\frac{1}{2}$ hours, and about 19 hours. He interpreted these findings in terms of cycles of virus growth, of which the first cycle lasted longer than succeeding cycles. However, it was later found that infected cells continue to liberate influenza virus over long periods of time

(Henle *et al.*, 1954). In addition, Schlesinger and Karr (1956) observed that in a similar system, when cells were infected at a high multiplicity, i.e., when presumably all cells were infected initially, periodic increases and decreases occur in the amount of liberated virus, associated with stepwise breakdown and partial restoration of the inhibitor of viral hemagglutination present in the chorioallantoic membrane. These periodic increases and decreases appear to correspond in time to the cycles observed by Cairns (1952). Recently, Cairns (1957) has shown that there is asynchrony in the initiation of infection by influenza virus, with great variation in the time before hemagglutinin appears in the allantoic fluid after infection with very small inocula. This finding makes it even more difficult to disentangle the different cycles which together make up the normal growth curve.

Tyrrell (1955) found that when influenza virus was grown in tissue cultures of chick embryo lung the average yield of virus was 650 hemagglutinating particles per cell. In this technique one hemagglutinating particle corresponds roughly to one infective dose and 10 virus particles as counted in the electron microscope (Tyrrell and Valentine, 1957), i.e., the yield was about 6500 virus particles per cell. The period of liberation observed was 72 hours. Recently, Ledinko *et al.* (1957) studied the growth of influenza virus in trypsinized suspensions of chick embryo lung cells, adding RDE after the initiation of infection in order to restrict the growth of virus to a single cycle. After a lag period of $2\frac{1}{2}$–$2\frac{3}{4}$ hours there was an exponential rise in the titer of extracellular infective virus for about 2 hours, with a rate of increase of roughly 10-fold per hour.

D. Newcastle Disease Virus of Fowls (NDV)

Rubin *et al.* (1957) carried out one-step growth curve studies of NDV grown in monolayers of chick embryo lung epithelium, the cells being infected at a multiplicity of 2 infective doses per cell. The lag period, as measured by the first appearance of new virus, was 3–4 hours but the average latent period, defined as the time when the yield of virus corresponded to one infective particle per cell, was 5–6 hours. In studying the development of cell-associated viruses, the cells were treated with antiserum before disintegrating them by three cycles of freezing and thawing. The antiserum treatment was necessary to remove virus superficially adsorbed to the cells and to give a clearer picture of the development of intracellular virus. Intracellular virus was found to increase at a nearly exponential rate, with an approximately 30-fold increase between the 4th and 6th hours before the increase tailed off. The curve of extracellular virus increased more steeply and 24 hours after infection there were roughly 1000 infective doses of virus released per infected cell. About 50 % of the final yield of virus was produced at 8–10 hours after the initiation of infection.

In selecting studies of viral growth to quote in this section an attempt was made to choose experiments in which either a one-step growth curve was carried out or some other method was used for restricting the viral growth to a single cycle of multiplication. For herpes simplex and vaccinia viruses, however, this has not been possible and values quoted for the rate of growth and yield of these viruses are subject to the qualification that growth of virus is accompanied by spread of newly formed virus to fresh cells with secondary and later cycles of virus growth.

E. Herpes Simplex Virus

Scott *et al.* (1953) studied the growth of herpes virus on the chick chorion. After a lag period of 6 hours the virus increased exponentially until about 16–18 hours, when a plateau was reached; this was followed by another slow rise from 24 to 48 hours. The smaller the inoculum the longer was the lag period but the rate of viral increase was independent of the size of the inoculum, being about one $\log_{10}$ every 2 hours. During the period of rise of infectivity in the membranes there was a parallel rise of infectivity in fluid washings (i.e., extra-cellular virus), but the titer in the washings was about 1 $\log_{10}$ less than in the membrane. At 24–48 hours the titer in the washings was about the same as that in the membranes. The results quoted by Wildy (1954) follow closely those of Scott *et al.* After a lag period of about 6–8 hours there was an exponential increase of cell-associated virus with an increase of about 3 $\log_{10}$ over a period of about 10 hours; again the extracellular virus lagged considerably behind the cell-associated virus. The presence of "steps" in the growth curve, as suggested by Wildy, is not very clearly defined.

F. Vaccinia Virus

Briody and Stannard (1951) measured growth curves of vaccinia virus on the chick chorioallantoic membrane. They thought that steplike increases in virus activity occurred at 8 and 16 hours after infection, but the findings were not consistent or in line with those of other workers. Anderson (1954), working with the same system, noted a steady decrease in the amount of recoverable virus up to the 9th hour. Thereafter, the amount of virus in the membrane increased exponentially at a rate of about one $\log_{10}$ every 4 hours. Overman and Tamm (1957) studied the development of vaccinia virus grown in pieces of chick chorioallantoic membrane *in vitro*. After a lag period of about 8 hours the virus increased at a nearly exponential rate until the 48th hour. However, in this system the growth of virus was much slower than when virus was grown *in vivo* and the increase was only about 2 $\log_{10}$ over the 40-hour period. The maximal virus yield in the membranes occurred at 3 days. The titer of virus in the medium was always much less than that in the

membrane at the same time, the proportion being about 1 % at 3 days; even at 7 days it was less than 10 %. Maitland and Magrath (1957) have also studied the growth of this virus in pieces of chorioallantoic membrane suspended *in vitro*, as well as in minced chick embryo and chick embryo cell suspensions. The increase in infectivity was variable from one experiment to another but tended to be more nearly exponential than linear.

In reviewing studies of growth curves for such different viruses grown under varying cultural conditions it is surprising to find so many similarities. After a lag period, all the viruses studied have shown an *exponential* increase in titer for part of their growth cycle. Also, there is a suggestion that those viruses which have a longer lag period have a slower rate of increase of viral infectivity. It is surprising, too, how frequently a rate of increase of infectivity of the order of about one $\log_{10}$ per hour has been reported, i.e., for poliomyelitis (Dulbecco and Vogt, 1955), western equine encephalitis (Rubin *et al.*, 1955), influenza (Henle *et al.*, 1947; Ledinko *et al.*, 1957), and Newcastle disease virus (Rubin *et al.*, 1957). It is interesting to note that all these viruses contain ribonucleic acid (RNA); on the other hand, herpes simplex and vaccinia, probably both DNA viruses, seem to have a longer lag period and a slower rate of growth. It is worth speculating whether the rate of growth of these different viruses may depend on the rate of synthesis of the different nucleic acids in infected cells.

V. The Release of Virus from Infected Cells

It is striking in comparing growth curves for different animal viruses to note great differences in the ratio of extracellular to cell-associated virus titer during the period of exponential increase of virus, e.g., for western equine encephalitis virus the extracellular virus titer was ten times greater than the cell-associated virus titer, whereas for vaccinia virus the extracellular virus was only 1/100 of the titer of cell-associated virus. These differences clearly reflect major differences in the rate at which fully infective virus is released from cells after it is formed. In this section the rate of release of different viruses is compared in order to gain some insight into the mechanism by which virus is liberated from infected cells.

A. Western Equine Encephalitis and Poliomyelitis Viruses

In studying the release of western equine encephalitis virus from individual cells, Dulbecco and Vogt (1954) found that by 4 hours, 60 % of cells had released small amounts of virus, i.e., were low yielders, whereas by 7 hours, only 11 % of the cells were low yielders, the other cells now giving high yields of virus. This finding implies that cells were continuing to release virus over the period 4–7 hours, i.e., individual cells release virus over a long period

of time. Rubin *et al.* (1955) tried to measure the release time for this virus. They assumed that the rate of increase of extracellular virus is at any instant proportional to the concentration of the intracellular virus present at that instant and to a velocity constant. This can be expressed as $dc/dt = K_1B(t)$, where K_1 represents the probability per unit time that an intracellular virus particle will be released. (It is, however, theoretically possible that K_1 might alter during the growth cycle, if for example, the release of virus was accelerated as cell damage increased.) Therefore, $1/K_1$ is the average time for virus to be released once it has become infective, and it can be calculated either mathematically or graphically from the experimental data on the rate of increase of intracellular and extracellular virus. For western equine encephalitis virus growing in chick embryo monolayers the average release time was calculated to be just under one minute.

Lwoff *et al.* (1955) noted an extremely rapid release of poliomyelitis virus from individual monkey kidney cells, practically all the virus being released within half an hour. On the other hand, Howes and Melnick (1957) concluded that Type I poliomyelitis virus was slowly released from monkey kidney monolayers. This is based on the findings that the increase in extracellular virus began about one hour after the increase in cell-associated virus and that the free virus was always much lower in titer. Even after 11 hours' growth, less than 20 % of the total virus was free. These findings are very different as regards extracellular virus from those of Dulbecco and Vogt (1955), who measured the rate of increase of extracellular (but not cell-associated) virus in the same cells. The only obvious difference between the two sets of experiments is that Dulbecco and Vogt used a one-step growth curve technique in order to minimize adsorption of newly released virus to fresh cells, whereas Howes and Melnick assayed samples from the monolayers directly, where presumably newly released virus might be rapidly adsorbed to fresh cells. Possibly, therefore, rapid release of virus is a more accurate picture for poliomyelitis virus, but direct comparison of the two techniques might help to decide this point.

B. Myxoviruses

Cairns (1952) studied the release of influenza virus from eggs infected with virus at limiting infective dilution, where presumably a single allantoic cell was infected initially. He found that virus liberation occurred over a period of 3 hours. The technique was not suitable for measuring further liberation of virus since additional cycles of infection could not be prevented. However, Henle *et al.* (1954) inoculated de-embryonated eggs with 10^6 ID_{50} of influenza virus and measured the differential (hourly) release of virus.

RDE was given with each hourly washing in order to restrict virus liberation to a single cycle, a finding which they demonstrated experimentally. It was found that virus liberation occurred at a constant rate for 36 hours, so that the virus must leave the cells by a process which does not destroy the cells.

Cairns and Mason (1953) measured the amount of influenza hemagglutinin in chorioallantoic membrane and allantoic fluid at hourly intervals after infection with very large doses of virus and found that between the 4th and 8th hours the membrane titers were consistent with a constant release period of about one hour. The experiments were then repeated but with large doses of RDE given $2\frac{1}{2}$ hours after the virus. No hemagglutinin was detectable in the membrane until 7 hours after infection and thereafter the titer was about one-tenth of that in the fluid. The effect of the RDE was therefore to elute newly released virus from the surface of the cells, and Cairns and Mason calculated that the liberation time, excluding the time during which virus can be released by the action of RDE, is less than 2 minutes. The most obvious explanation of these findings is that once it is formed the viral hemagglutinin is rapidly liberated from the cells, but that it remains adsorbed to the cell surface, from which it is released within about one hour, presumably by its own enzymatic action. Support for this theory comes from the work of Ackermann and Maassab (1954). They found that the release of influenza virus from chick chorioallantoic membrane *in vitro* was inhibited by α-amino-*p*-methoxyphenylmethane sulfonic acid (AMPS), and that this effect was reversible by RDE given after the virus. When concurrent titrations of virus in fluid and membrane were carried out, it could be shown that the effect of the AMPS was to delay by many hours the liberation of virus from the cells. Ackermann and Maassab interpret these results as indicating that normally the viral enzyme functions in liberating virus from the cells and that AMPS inhibits the enzymatic activity of the virus, the action of AMPS being in turn reversible by RDE.

Rubin *et al.* (1957) calculated the release time for Newcastle disease virus grown in chick embryo lung epithelium, using the same assumptions and formula as these workers had adopted in their work on western equine encephalitis virus. The average release time for NDV was found to be 80 minutes. However, by treating the cells with NDV antiserum before disrupting them, it was found that a great deal of cell-associated virus could be neutralized during the period of exponential rise, although antiserum treatment had little effect when carried out during the lag period. This suggests that during the period of exponential rise in virus titer most of the cell-associated virus is superficially adsorbed to the cell surface; when the release period was recalculated to take this into account, it was found that only a few minutes were required for newly matured virus particles to reach the cell

surface. The results are strikingly similar to those obtained by Cairns and Mason for influenza virus.

C. Herpes Simplex and Vaccinia Viruses

In their studies of herpes simplex virus, both Scott *et al.* (1953) and Wildy (1954) noted that the titer of released virus was about one $\log_{10}$ less than that in the cells during the period of exponential increase in virus titer. Similarly, for vaccinia virus, Overman and Tamm (1957) found that during the period of exponential increase less than 1 % of the virus in the membranes was released into the medium. It is clear, therefore, that herpesvirus and vaccinia viruses, once formed, are only slowly released from the cells. Calculation of the release time would not be meaningful, however, since pock-producing viruses such as these probably spread from cell to cell without being liberated into the medium. Andrewes (1930) showed that herpesvirus could continue to grow in tissue culture in the presence of immune serum, which, however, prevented the initiation of infection and Black and Melnick (1955) noticed that herpes B virus grown in monkey kidney cells was able to form plaques even without an agar overlay. Primary plaques could not be prevented from developing by immune serum given shortly after the virus but the serum prevented the appearance of further plaques. By contrast, poliomyelitis virus grown in the same cells did not form plaques and immune serum could avert the spread of the virus even when infection was well under way. From what is known of influenza virus, it behaves very like poliomyelitis virus in this respect. We have, therefore, two contrasting modes of virus release and spread. In the first, exemplified by poliomyelitis, virus is rapidly released from cells once it is formed and spreads to new cells mainly by invasion via the medium. In the second, exemplified by B virus, virus is slowly released from cells once it is formed and can spread to neighboring cells by direct cell-to-cell spread. Cell-to-cell spread presumably occurs with varicella virus too, since Weller (1953) found that passage could be effected only by cell extracts and not by the fluid phase. For four different adenoviruses, Ginsberg (1957) has also provided evidence of slow release of virus from these cells. Once more, it is interesting to note that the division of animal viruses into those which are released rapidly from cells and those which are released more slowly corresponds (so far as it is known) to viruses which contain RNA and those which contain DNA. A possible division in terms of size alone is ruled out by the fact that the adenovirus, a DNA virus, is slightly smaller than influenza virus. At the moment, there is little guide to the possible significance of this observation, and, indeed, it is difficult to be sure of its accuracy, since the different viruses have not been studied under comparable conditions. However, this may be an interesting generalization and it seems important to see how far it is applicable among the animal viruses.

D. Mechanism of Release of Virus and Virus Filaments

During the multiplication of bacteriophages release of virus occurs when the bacteria lyse. The results quoted in the previous three sections show that for most animal viruses virus release can occur without cell lysis. Nevertheless, there is a range of behavior from a virus such as western equine encephalitis virus, which is very rapidly released from cells within a minute or two of its maturation, to a virus like that of varicella in which release is minimal under ordinary conditions of culture, and spread mostly occurs directly from one cell to its neighbors. Further details of the mechanism of virus release are best investigated by morphological studies; these are reviewed in Chapter 5. In passing, however, it may be noted that there is some morphological evidence to justify the biological findings on virus release for different viruses. Thus, Lwoff *et al.* (1955) studied the appearance of single cells by phase-contrast microscopy in parallel with biological observations on the release of poliomyelitis virus. A hyaline zone appeared at the periphery of the cell just before virus release occurred and underwent a pronounced vacuolization during the time of release. This suggests that release of virus occurs through lysis of part of the cell and that there was extensive cell disruption by the time virus release ended. On the other hand, Robinow (1950) found that vaccinia virus was excreted from cells along narrow filaments of cytoplasm protruding from the cells. The virus appeared to pass to the tip of these stalks and then to leave the cell with only minimal damage to the cell surface. There is a further contrast in a virus like adenovirus, which Morgan *et al.* (1956a) demonstrated in their beautiful electron micrographs to be almost exclusively intranuclear, and in influenza virus particles, which could be found just beneath the cell surface only (Morgan *et al.*, 1956b). Presumably, influenza virus particles, in contrast with adenoviruses, are finally assembled just beneath the cell surface and are excreted from the cell soon after they are formed.

From the biological point of view, one of the most interesting phenomena bearing on the question of formation and release of virus particles is the occurrence of filamentous forms of virus. Since they were first described by Mosley and Wyckoff (1946) filaments have been found associated with almost all members of the myxovirus group, but they are most characteristic of recently isolated strains of influenza virus A (Chu *et al.*, 1949). The evidence obtained by Donald and Isaacs (1945b) from particle counts and infectivity titrations and on the effect of ultrasonic vibrations suggested that filaments were infectious, and that they could be fragmented into many hemagglutinating segments but without increasing the number of infective units. Burnet (1956) found that filaments could be ruptured by suspending them in water without reducing significantly the infectivity of the preparation; he suggested that infectivity was limited to the sphere frequently found at the tip of the filaments. Burnet also noted that filaments had some of the physicochemical

characters of the cell surface although their serological behavior is that of virus, since they are specifically agglutinated by viral antiserum. Valentine and Isaacs (1957) observed in an electron microscopic study that, on digestion with acid and trypsin, filaments largely disappeared, whereas trypsin-resistant nucleoprotein rings remained after similar treatment of virus spheres; Ada and Perry (1958) and Burke *et al.* (1958) found that filamentous preparations of influenza virus contained much less ribonucleic acid than the corresponding spherical forms. Taken together, these results point to filaments having an infective "warhead" (Lindenmann, 1957) containing nucleic acid, and a long tail containing noninfective viral hemagglutinin.

Morphological studies of filaments in the course of formation and cut in ultrathin sections show that the interior of the filament is continuous with the cytoplasm of the cell (Morgan *et al.*, 1956b), and that the surface of the filament is continuous with the surface of the cell (Bang and Isaacs, 1957). The conclusion suggested by these findings is that viral spheres forming near the cell surface may drag out long filaments of the cell cytoplasm behind them; on the basis of Cairns and Mason's work this process may occur during the time that the virus is trying to free itself by its enzymatic action from mucoprotein present at the cell surface. At some point, the filaments would become nipped off at their base and so be formed of variable length. If this is so, one must conclude that a large part of the cytoplasm near the surface of the infected cell has acquired the specific hemagglutinating and serological behavior of virus elementary bodies (see Section VII). It seems, too, that highly filamentous strains of virus cause little damage to the cell surface. On the other hand, as the virus becomes better adapted to a tissue, filaments become much less common and it may be that the process of adaptation is accompanied by an increasing ability of the virus to damage the cell surface, thus preventing the formation of filaments. In this connection, Bang (1955) has illustrated the different degrees of cell destruction produced by virulent and avirulent forms of virus. Finally, one cannot but be struck by the almost haphazard way in which influenza virus filaments and spheres appear to be assembled together and released from the cells, a picture which contrasts strikingly with the regular array of adenovirus particles demonstrated by Morgan *et al.* (1956a) within the cell nucleus.

VI. Incomplete Virus

In the previous sections of this chapter different intracellular stages in the normal cycle of viral development have been described. This final section is devoted to the abnormal development of virus which occurs under certain conditions, particularly when cells are heavily infected with virus, and which results in the formation of what von Magnus called incomplete virus. There

are still two possibilities with regard to incomplete virus. It may be thought of as deformed virus, produced as a result of a faulty cycle of multiplication, or it may be immature virus prematurely released from cells before its development had been completed. At the moment, it seems to be logically impossible to distinguish between these two possibilities, and it may be best to regard incomplete virus simply as the product of an abnormal cycle of virus development without specifying the abnormality further. Unfortunately, most of the work on incomplete virus has been carried out with influenza viruses, although recently there have been hints that the same phenomena may apply to other viruses. The formation of incomplete virus is such an interesting phenomenon that search among other viruses seems well worthwhile.

A. Properties of Incomplete Virus

Incomplete influenza virus, as defined by von Magnus (1946), and prepared by serial passages of undiluted allantoic fluid virus, has a very low infectivity relative to its agglutination titer. In comparison with standard influenza virus, incomplete virus may have a ratio of infectivity/hemagglutinin titer (I/HA ratio) of 10^{-1} to 10^{-5} of that expected. The hemagglutinating behavior of incomplete virus does not differ from that of standard virus as determined by the number of virus particles (seen on electron microscopy) per agglutinating dose (Werner and Schlesinger 1954; Donald and Isaacs 1954a). Hence, the characteristic property of incomplete virus is its low infectivity per virus particle.

In addition to its low infectivity, incomplete virus is also reported to have a low toxicity when injected intracerebrally into mice (Bernkopf, 1950). However, it appears to be a good interfering agent, as shown by its ability after inoculation into the chick embryo to suppress hemagglutinin production by a large dose of standard influenza virus given 18 hours after the incomplete virus (von Magnus, 1954). These findings are much more significant than earlier results reported by von Magnus (1951a) of "autointerference" induced by incomplete virus inoculated intranasally into mice, since the interpretation of autointerference in mice is complicated by the fact that the incomplete virus would provide an antigenic stimulus which might mimic interference. This possibility can be inferred from further experiments by von Magnus (1951a) from which it is clear that incomplete and standard influenza virus showed very similar antigenic behavior, both in *in vitro* hemagglutination inhibition tests and when formolized and tested for their antigenicity in mice. In the latter case, the antigenicity of the two preparations was proportional to their agglutination titer (i.e., particle count) and not to their infectivity. This finding implies that incomplete and standard virus may have a similar surface structure; further evidence for this came from experiments indicatin

that both viruses showed similar behavior on adsorption to, and subsequent elution from, chick red cells. In addition, Svedmyr (1949) found similar enzymatic activity of both forms in their ability to destroy the inhibitor present in normal allantoic fluid. From present information, therefore, incomplete and standard virus have a similar surface structure and the difference between them is a more fundamental one.

One difference which was noted early in a careful comparison of standard and incomplete virus was in their sedimentation behavior in the high-speed centrifuge (Gard *et al.*, 1952). Standard virus consisted for the most part of rather homogeneous particles with a sedimentation constant of 747 A. In highly incomplete virus preparations the 747 S component was replaced by a very inhomogeneous slower sedimenting component of sedimentation constant varying from 430 to 675 S. As mentioned earlier, there was not sufficient morphological difference between particles of standard and incomplete virus seen in electron micrographs to account for the different sedimentation behavior, and a difference in particle density appeared to be the most likely explanation. Support for this came from a study by Uhler and Gard (1954), who found that incomplete virus had a higher lipid content than standard virus. From a preliminary analysis of the lipid content of preparations showing varying degrees of incompleteness, it was concluded that the findings could not be explained by postulating virus of low and high lipid content, i.e., there was some variability in the lipid content among different virus particles. These findings might, therefore, account for the unusual sedimentation behavior of incomplete virus, but, at the moment, there is no explanation for the difference in lipid content in terms of mechanisms of viral synthesis, nor is it clear what is the influence, if any, of the lipid content of the virus particle on its infectivity.

More pertinent to the problem of the infectivity of incomplete virus is the work of Ada and Perry (1956) on the nucleic acid content of preparations of influenza virus showing varying degrees of incompleteness. In a most important study, these workers showed that when the infectivity of different preparations (expressed as log infectivity titer/agglutination titer) was plotted against its nucleic acid content a linear relation was found. There was no simple proportionality between infectivity and nucleic acid content and a hundred-fold decrease in infectivity was accompanied by a drop in RNA content from about 1 % to 0.5 %. It is interesting to note that Lief and Henle (1956b) found a very similar ratio between the amount of "soluble antigen" (see Section III, A) which could be extracted from incomplete virus by ether treatment and the 1/HA ratio of the virus used, i.e., there appears to be a close relationship between the soluble antigen content and the nucleic acid content of the virus. The proportions of the nucleotide bases in the study by Ada and Perry were not significantly different in incomplete and

standard virus. These findings imply that preparations of virus showing varying degrees of incompleteness are heterogeneous with regard to the total nucleic acid content of their individual virus particles. In view of the known significance of nucleic acids in the infectivity of bacteriophages and tobacco mosaic virus, it seems reasonable to conclude from Ada and Perry's findings that the probability of a virus particle being able to initiate infection may be a function of its nucleic acid content. This conclusion gains further significance when we consider the mode of production of incomplete virus.

B. Production of Incomplete Virus

The all-important factor in producing incomplete virus seems to be that large doses of seed virus are essential. As is discussed below, seeds obtained after two, three, or more serial passages of undiluted virus are more effective in producing incomplete virus than seed which has not been passaged in this way. Nevertheless, the same fact holds with all these seeds, i.e., if they are passaged in undiluted form, further incomplete virus is produced, whereas if they are passaged diluted 1/100 or more, standard virus is once more produced (von Magnus, 1951c). It is natural to suppose that the critical factor in producing incomplete virus is the multiplicity of infection. This point was first discussed by von Magnus (1951c) who found that about 10^2 agglutinating doses of virus, or more than 10^9 virus particles (Donald and Isaacs, 1954a) was the minimum amount which would produce incomplete virus. Recent counts of the number of surface allantoic cells in 10- and 11-day eggs are between 1.8×10^7 (Tyrrell *et al.*, 1954) and 3 to 4×10^7 cells (Cairns and Fazekas de St. Groth, 1957); this would correspond to a multiplicity of between about 10 and 100 virus particles per cell. Since incomplete virus is not produced when seeds are diluted 1/100 or more, these figures support the idea that multiple infection of cells is an important factor in the production of incomplete virus. On the other hand, Cairns and Edney (1952) concluded from their results that incomplete virus was produced when only 1 % of the cells was infected initially. However, their calculations were based on the assumption that a multiplicity of 1 occurred when $10^{2.2}$ agglutinating doses of virus were taken up by the allantoic cells; more recent techniques of counting virus particles and allantoic cells would suggest a much higher figure for the multiplicity after this inoculum. From Cairns and Edney's experimental findings, incomplete virus production is first detectable when between 10 and 100 agglutinating doses of virus are taken up (10^8 to 10^9 virus particles) and the corresponding figure given by Horsfall (1954) is 3×10^7 "hemagglutinating" particles, which is equivalent to 3×10^8 virus particles (Tyrrell and Valentine, 1957). There is, therefore, nothing in these estimates to refute the idea that multiple infection of cells is a critical factor in producing incomplete

virus. Since a low nucleic acid content appears to be the characteristic deficiency of incomplete virus, it seems that multiple infection may strain the cells' ability to synthesize viral nucleic acid, a hypothesis which might be tested experimentally.

The second factor which von Magnus (1951b) stressed was that the degree of incompleteness increased with serial passage. In a most detailed series of growth curves he studied the effect of serial passages of PR8 virus in the undiluted form. In the standard virus used to initiate the passages the ratio of infectivity : hemagglutinin titer (I/HA ratio) was about 10^6. First passage virus had an I/HA ratio of about 10^5; second passage, about 10^3; third passage, between 10^1 and 10^2; and fourth passage, between 1 and 10. Since no attempt was made to wash out the inoculum, the infectivity of the residual inoculum may have accounted for a proportion of the infectivity found, so that the I/HA ratios might have been even lower had the inoculum been removed before the new yield of virus was liberated. This may explain the finding that, in the de-embryonated egg, where the inoculum is removed, there is more incomplete virus produced in a single passage (Bernkopf, 1950), and the same is true in the intact egg (Cairns and Edney, 1952). Another finding which von Magnus (1951b) noted was that while the I/HA ratio declined on passage, the yield of hemagglutinin was not greatly affected until the fourth passage, when it was one-tenth (or less) of that produced in standard passages. As a result, when a fifth passage was made with the same volume of infected fluid, the number of particles present was considerably less than in earlier passages and, as would be expected on the hypothesis that multiplicity of infection was a critical factor, the I/HA ratio of fifth passage material again rose sharply. The low yield of hemagglutinin in fourth passage material is presumably a manifestation of viral interference, but since interference requires many hours to become established (Fazekas de St. Groth *et al.*, 1952), it is only apparent in these experiments when the amount of infective virus in the inoculum is very small. Experimentally, von Magnus (1954) has demonstrated that a large inoculum of incomplete virus suppressed hemagglutinin production by a large dose of challenge virus, provided a few hours' interval was allowed before the challenge virus was inoculated; an 18-hour interval gave the highest degree of interference. The serial passage experiments thus disclose a complex situation. As a result of multiple infection of cells with standard virus particles, the progeny has a low I/HA ratio and a low nucleic acid content, and on serial passages this process is continued. Experimental addition of about 10^6 ID_{50} of standard virus along with incomplete virus did not significantly influence the yield from the incomplete virus alone; large doses of standard virus led to a slightly greater yield of virus, but with a lower I/HA ratio than when the same dose of standard virus alone was given (von Magnus, 1951c). Hence, when the incomplete virus particles infect a cell at the

same time as standard virus particles, there is a sharing of the available nucleic acid among the progeny. If the incomplete virus is given sufficiently long before the standard virus, there is time for viral interference to become established and a diminished yield of total virus results. The suggestion that in multiple infection of cells the nucleic acid may be shared among the viral progeny is intended as an analogy to ideas of viral recombination.

Another method of producing incomplete virus is to use as seed standard virus which has had its infectivity greatly reduced by incubation for a few days at 37 or 22°C. (Henle, 1953; Horsfall, 1954; Paucker and Henle, 1955a). In an experiment described by Paucker and Henle (1955a), a preparation of virus with an I/HA ratio of $10^{6.1}$ was incubated at 37°C. for 5 days, when its infectivity had dropped more than 100,000-fold, the I/HA ratio being $10^{0.9}$. When this was used undiluted as seed the progeny had an I/HA ratio of $10^{2.3}$; seed diluted 1/10 gave progeny with an I/HA ratio of $10^{2.9}$, while seed diluted 1/100 gave progeny with an I/HA ratio of $10^{6.4}$. These findings show that hemagglutinating virus of low infectivity is produced by seed virus which has been inactivated at 37°C.; there is a striking resemblance to the production of incomplete virus by von Magnus's method in that only concentrated inocula produce this effect, while on dilution of the seed 1/100 standard virus is produced. One difference is that when incomplete virus is prepared by von Magnus's method, passage of incomplete virus results in progeny with a lower I/HA ratio, whereas inoculation of virus inactivated at 37°C. leads to progeny with a slightly higher I/HA ratio than the seed. This finding argues against the possibility that incomplete virus formation in von Magnus's experiments is caused by accumulation of virus spontaneously inactivated at 37°C., as does the fact that the incomplete virus released in the first 2 hours of the growth cycle has a low I/HA ratio, similar to that formed later in the cycle (Finter *et al.*, 1955). At the moment, there is no evidence on the nucleic acid content of progeny from virus inactivated at 37°C.; this would be an interesting point to investigate. If the nucleic acid were low, the results might be explained by postulating that during prolonged incubation at 37°C. the virus nucleic acid becomes sufficiently damaged to prevent the normal synthesis of viral nucleic acid, but, provided multiple infection of cells occurs, it is still able to take part in the synthesis of a lower quota of nucleic acid with a resulting yield of incomplete virus.

The finding by Fazekas de St. Groth and Graham (1955) that, in eggs treated with metaperiodate, influenza virus growth results in the production of hemagglutinating virus of low infectivity may be due to the action of aldehydes on the virus rather than to an action on the cells, since a similar effect could be demonstrated on virus *in vitro* (Liu *et al.*, 1956; Schlesinger and Karr, 1956). Fazekas de St. Groth and Graham (1954) also investigated production of incomplete virus by different strains of influenza virus, using

the technique of serial passages (von Magnus, 1951b). They found that different strains varied in the ease with which they could be induced to form incomplete virus. When the strains were arranged in order of ease, the only biological activity with which the order corresponded was the time taken for effective entry of virus into the cell. At the moment, the significance of this interesting observation is not known.

C. Partial Cycle of Virus Development Produced by Incomplete Virus

In earlier work on incomplete virus it was assumed that the incomplete virus was noninfective, but more recently it has become clear that it can initiate partial cycles of virus development. Burnet *et al.* (1954, 1955) carried out some very interesting studies of the growth of incomplete virus in de-embryonated eggs. The technique used was to inoculate different doses of incomplete virus, remove the seed by washing, and treat the membranes with RDE in order to obtain a single cycle of growth. The fluids were then harvested at 7 hours and the yield of hemagglutinin titrated; in addition, the amount of hemagglutinin produced between 7 and 22 hours was measured. Over a given range of virus dosage the yield of hemagglutinin at 7 hours was proportional to the amount of virus taken up; this applied to both complete and incomplete virus. This finding shows that in both cases a single cycle of virus production was being observed. However, the yield from incomplete virus was much greater than would have been expected from the infectivity of the inoculum; in one experiment, quoted by Burnet *et al.* (1954), the yield from an incomplete virus preparation with a I/HA ratio of $10^{-3.1}$ of the complete virus used gave a yield of one-sixth of the complete virus at all virus dosages tested. Since the yield per particle was independent of the virus dosage and on the basis of calculations of the multiplicity of infection the results could not be explained by multiplicity reactivation. Burnet *et al.* conclude that a considerable proportion of the incomplete virus in the seed is able to undergo a single incomplete cycle of multiplication to produce viral hemagglutinin, but that this hemagglutinin is unable to continue to produce full infection. Paucker and Henle (1955b) have also found that virus heated at 37°C. and rendered noninfective was still able to produce hemagglutinin in a single cycle. They suggested that the live virus particles which remained in the seed after inactivation at 37°C. were able to initiate a single cycle of virus growth, but were prevented from initiating a second cycle by viral interference induced by the inactivated virus in the seed; as mentioned earlier (Section VI, B), this interference would not be established immediately and hence there would be no inhibition until the first cycle of virus growth had been completed. In the experiments of Burnet *et al.* (1954) the restriction to a single cycle of virus production at high virus doses may also be due to a late induction of interference.

Beale and Finter (1956) studied the ability of preparations of incomplete virus to produce soluble antigen in the chorioallantoic membrane after inoculation into the allantoic cavity. They measured the appearance of soluble antigen 6 hours after inoculation and compared the amount found with that produced by corresponding amounts of standard virus when similarly inoculated. It was found that preparations of incomplete virus produced much more soluble antigen in this test than would have been expected on the basis of their infectivity, although less was produced than by the same number of standard virus particles (assessed by the hemagglutinin titer). In these respects the findings are strictly analogous to those of Burnet *et al.* (1954) on the production of viral hemagglutinin by incomplete virus. Furthermore, as a parallel to the findings of Paucker and Henle (1955b) on the production of hemagglutinin by virus inactivated at 37°C., Isaacs and Fulton (1953) found that virus inactivated by heating at 56°C. for 8 minutes produced significantly more soluble antigen when grown on the chick chorion than would have been expected from the infectivity of the inoculum. Beale (1954) has also found that the virus present in the chorioallantoic membrane 4½ hours after inoculating a large dose of influenza virus has a low I/HA ratio, and that this, too, when examined by the 6-hour soluble antigen test, produces more soluble antigen than would be expected on the basis of its infectivity. Presumably, this is another manifestation of the behavior of incomplete virus, in this case present in the chorioallantoic cells 4½ hours after inoculation and before being liberated. However, a precursor to fully infective virus might be present in such membrane extracts and it would be interesting to test the behavior of the "S" hemagglutinin in experiments of this kind.

Incomplete influenza virus is not wholly noninfective, therefore, but is capable of initiating a partial cycle of virus multiplication, though not a complete cycle. In addition, virus which has been inactivated by heating at 37°C., or 56°C. for a short period, behaves in a way which is superficially similar to incomplete virus in this respect. It appears that virus which lacks its normal complement of nucleic acid, although unable to complete the virus multiplication cycle, may nevertheless initiate the synthesis of some viral constituents.

D. Incomplete Viruses Other Than Influenza

Although the formation of incomplete viruses is an important phenomenon to understand in itself and also because of the light it throws on virus multiplication processes in general, almost all the work on the subject has had to be carried out with influenza viruses. Even such closely related viruses as NDV and fowl plague apparently do not give rise to incomplete virus when passaged by similar techniques to those used by von Magnus for influenza,

and outside this group of viruses there are very few published accounts of attempts to prepare incomplete virus forms. This seems a pity, since the phenomenon is most interesting and information on the conditions under which it is applicable to other viruses would be worth having.

Mims (1956) described some indirect evidence that Rift Valley fever virus could be induced to show incomplete virus forms. Mims noted that when large inocula of virus were passaged serially in mice low infective titers resulted, whereas high titer virus was produced by small inocula. The low-infective virus was shown to be capable of interfering with the growth of infective virus by prolonging the incubation period and reducing the peak infectivity titer reached. However, these effects could be explained in alternative ways and, in particular, there is a possibility that if the seed population of virus particles is heterogeneous in respect of virulence, large doses of virus may act as an antigenic stimulant and produce an apparently similar effect (cf. Schlesinger, 1949).

Sanders (1957) has shown that encephalomyocarditis virus can be titrated in three different ways. The plaque-forming titer measures the infectivity of a preparation in a plaque titration with ascites tumor cells of mice. The same cells can be used to measure a second property of the virus, its cell-killing ability; this depends on the fact that aqueous eosin stains dead cells and the virus can be titrated indirectly by this method by calculation from the number of unstained cells (the calculation is based on the Poisson distribution). Finally, the virus can be titrated by its hemagglutinin titer, and there is good evidence that all three titrations measure properties of the virus particles themselves. If virus is incubated for more than 1 hour at 37°C., the plaque-forming titer is reduced more than is the titer by the other methods, and the same is true of virus freshly released from cells after infection with high multiplicities of virus. This phenomenon requires further investigation, but the results of these experiments seem to resemble closely those of von Magnus for influenza virus. Recently, Cooper and Bellett (1957) have produced some evidence that an autointerference phenomenon observed with vesicular stomatitis virus grown in chick embryonic cells *in vitro* is due to the production of incomplete forms of virus. The incomplete, or T, forms of virus are produced on serial undiluted passages, but not with diluted passages, and only when cells are mixedly infected with T and live virus particles; also the T forms are spontaneously liberated from cells, as in the case of influenza virus.

VII. Conclusions

Throughout this chapter, the attitude adopted has been that although research on the multiplication of bacteriophages has provided the greatest stimulus to the investigation of similar problems in animal viruses, in all

consideration of work on animal viruses the results should be judged on their own merits. Clearly, there are great differences between the multiplication of animal and bacterial viruses, particularly in the way they penetrate and are released from their host cells. Nevertheless, such differences are quite overshadowed by the many strong resemblances found in their methods of multiplication. More particularly, in plant, bacterial, and animal viruses more and more evidence is accumulating to show that the key role in multiplication is played by the viral nucleic acid. Some recent work from Tübingen on animal viruses adds emphasis to this conclusion. Wecker and Schäfer (1957b) examined chick embryonic cells during the lag period, i.e., 30–180 minutes after infection with P^{32}-labeled fowl plague virus. They found that in extracts of these cells a high proportion of the radioactivity was not sedimented under conditions when the virus elementary bodies would have been sedimented. From such extracts a proportion of the radioactivity could be precipitated by antiserum to the soluble antigen and part of the remainder was shown to consist of viral nucleic acid by the fact that it was hydrolyzed by ribonuclease. It seems, therefore, that after the virus particles enter the cells many of them are broken down to give soluble antigen (a ribonucleoprotein) and free nucleic acid, with phospholipid in addition. The further course of infection in a similar system was studied by Breitenfeld and Schäfer (1957) by means of antibody, labeled with fluorescin isocyanate, to both soluble antigen and hemagglutinin. This technique of localizing antigens within cells was preferred to that of homogenization and differential centrifugation, since it was found that by the latter method viral constituents were nonspecifically adsorbed to the various cell components. They found that soluble antigen was first detected at 3 hours after infection and was localized to the cell nuclei (cf. Liu, 1955). From 5 hours the fluorescence diffused slowly out of the nucleus in the absence of obvious nuclear damage, and by 14 hours the whole cell showed fluorescence. By contrast, the hemagglutinin was first detected at the fourth hour throughout the cytoplasm, but the nucleus was not stained. The hemagglutinin gradually moved toward the cell margin and at the fourteenth hour the cells showed deeply fluorescing borders. This is the first definite evidence that different viral constituents are synthesized in different cellular sites. Thirdly, Wecker and Schäfer (1957c) have now produced additional evidence that infection by eastern equine encephalitis virus can be initiated by viral ribonucleic acid. They extracted infected mouse brains with phenol and showed that these extracts were infective. Furthermore, in contrast to a preparation of virus with the same infective titer, the infectivity of the phenolic extracts was sensitive to very small amounts of ribonuclease (but not deoxyribonuclease); it was not sedimented significantly with high centrifugal forces; and it was not inactivated on precipitation with ethanol. There is, therefore, strong presumptive evidence that the infectivity

of these extracts is due to viral nucleic acid. When we remember, too, that in a number of animal viruses, as was shown for some plant viruses, the amount of ribonucleic acid in viruses of very different sizes is surprisingly constant, corresponding to a molecular weight of about two million per virus particle (Frisch-Niggemeyer, 1956), it is difficult not to feel that viral nucleic acids will all be found to play a similar role in other animal viruses. The difference between viral ribonucleic and deoxyribonucleic acids and the information contained in the nucleic acids which can induce the cell to synthesize viral constituents and assemble them into mature virus particles are surely the main challenges for future viral research.

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Chapter VII

Interference between Animal Viruses

R. Walter Schlesinger

Saint Louis University School of Medicine, Saint Louis, Missouri

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I. Introduction

Immunity to animal viruses is a peculiarly complex problem. More than any other phase of viral activity, it reflects the double standard of reference

under which work on viruses is carried out. The extracellular, mature virus particle is looked upon as an exogenous, infectious agent to which a complex host reacts much in the same way as to other parasites. On the other hand, once within the individual host cell, a viral particle behaves in the manner of an intracellular organelle, imposing on the cell new biological, metabolic, and genetic functions, including altered response to superinfecting virus. Thus, all conceivable components of the systemic immune response—specific or nonspecific—as well as strictly cell-bound, virus-induced modifications enter into the evolution of the immune state.

In this framework, viral interference occupies a special place. Its importance is underlined by the fact that mutual exclusion among phages and immunity of lysogenic systems are the only forms of acquired immunity found in infected bacteria, and that both depend entirely upon the modifying effect on the individual cell of a primary infection. Systemic immune mechanisms, so important in animals, do not enter into the picture. Yet, even within the narrow confines of the cosmos made up of *Escherichia coli* B and the T phages, the occurrence of mutual exclusion is subject to control by various factors. Among these are the relative serological and genetic compatibility of the two phages involved and variations in timing and dosage. Information obtained from studies on cell populations has been made more meaningful by analyses of single cell yields. Technically, it is possible to integrate bacterial immunity to infection as a logical phase into a unified concept of the virus-host relationship.

In the study of animal viruses, a unified concept of the infectious process is lacking. Hence, interference, even when observed under conditions not obscured by systemic immune mechanisms (i.e., in cell cultures or embryonic tissues), remains so far largely a descriptive term which cannot yet be defined in terms of its relation to the viral life cycle. The need for separate treatment of this subject is born of our ignorance.

In the following pages, we try to establish criteria toward a unified definition, collate many and diverse experimental observations, and summarize the meager evidence which may clarify the dynamics of the phenomenon but by no means the underlying mechanisms.

II. Definition of the Problem

A. Qualitative Criteria for a Definition of Interference

1. *The Relation to Virulence*

At the qualitative level, interference expresses itself as protection against injury, disease, or death, or, in general, against manifestations of "virulence." Unless the superinfecting virus is pathogenic for the host system involved, interference in this sense cannot be observed.

This restriction introduces into the concept of interference a complex and obscure parameter. Attempts to analyze the essence of virulence lead to the conclusion that it is an expression of multiple genetic factors. Recombination experiments with influenza viruses (Burnet, 1955, p. 431; Lind and Burnet, 1957b; Gotlieb and Hirst, 1954) indicate that the dosage of such factors is highly variable. Hence the phenotypic expression of the entire complex, virulence, is easily lost in recombinant progeny. The lability of this character has obvious relevance to our problem. Many studies of interference concern protection of the host or of cells against a virulent virus resulting from simultaneous infection with an avirulent variant of the same or a related agent. In interpreting such situations, we must consider the possibility that protection might in reality be due to loss of virulence in combinant viral progeny rather than to interference in the sense of mutual exclusion. The realization of this conflict will be reflected in the following pages, specifically in Section III, A, 2, *b*.

2. *Mutual Exclusion and Nonspecific Inhibition of Viral "Toxicity"*

The term "viral interference" conventionally has the connotation of mutual exclusion (Delbrück, 1945; Dulbecco, 1952) which, in turn, implies that superinfecting viral particles are somehow prevented from entering and multiplying in an already infected cell. In the case of bacterial viruses, however, a cell resistant to superinfection by virtue of mutual exclusion is not protected against "lysis from without" by an appropriate phage at high multiplicity (Doermann, 1948). In recent years, much evidence has accumulated for "toxic" cell damage by animal viruses (Cox, 1953) which is often independent of multiplication. What, if any, relation does protection or lack of protection against such effects have to interference?

The problem here is that protection can be achieved not only by pretreating the host with virus (Prince and Ginsberg, 1953; Fong *et al.*, 1953; Wagner, 1953; Groupé and Dougherty, 1956; Dougherty and Groupé, 1957; Manire, 1957; Herrmann *et al.*, 1955; Khoobyarian and Walker, 1957), but also with nonviral agents, such as Xerosin, bacterial vaccines, or heated receptor-destroying enzyme (RDE) (Groupé *et al.*, 1954; Groupé and Herrmann, 1955; Dougherty and Groupé, 1957; Wagner, 1953; Fong *et al.*, 1953; Ginsberg, 1955; Khoobyarian and Walker, 1957). Protection against toxic viral manifestations can be achieved without affecting viral multiplication (Groupé *et al.*, 1954b) or "incomplete" virus production (Frankel and Schlesinger, 1952; Ginsberg, 1955). Ginsberg has studied the effect of Xerosin on pneumonitis induced in mice by the CAM strain of influenza virus. The agent does not inhibit specific virus-induced injury to susceptible cells, but does prevent secondary reactions leading to pulmonary consolidation and death.

Studies along these lines confirm that viral multiplication and production of injury may be dissociated processes (Davenport, 1952). The protective effects of bacterial products are reminiscent of interference-like phenomena encountered in nonviral infections of vertebrates and invertebrates. Indeed, some of these are associated with markedly inhibited proliferation of the test pathogen. Thus, Parry (1956) found that inoculation of 10^3 organisms of a virulent strain of *Pasteurella pestis* killed mice more rapidly than did 10^6 organisms. He also showed that admixture of 10^7 avirulent organisms to 10^3 virulent ones gave complete protection, and that India ink had a similarly protective effect. Henderson *et al.* (1956) found that pre-existing respiratory infection with pathogens, such as *Brucella suis* or *Mycobacterium tuberculosis* protected guinea pigs against a different secondary invader given by the respiratory route. The phenomenon seemed to depend on the ability of both organisms to spread via the lymphatic system. If the latter was circumvented, no protection was observed.

Although the older and recent literature abounds with such reports, amazingly little is known about underlying mechanisms. Usually some alterations in the host's systemic defense apparatus are postulated which may involve phagocytes, the properdin system, or stimulation of certain enzymes (cf. report by Rowley (1956) on lipopolysaccharide-splitting phosphatase produced by animals injected with bacterial lipopolysaccharides). Undoubtedly, similar systemic effects can modify the response to pathogenic viruses and may be at the root of some of the observations referred to above. Be that as it may, the superficial resemblance of results like those of Parry and Henderson to autointerference and homologous or heterologous interference among viruses is sufficiently confusing to impose the need for restrictive criteria. In the qualitative sense, it is postulated that *interference results uniquely and directly from an association of the interfering virus with susceptible cells, and that this association is primarily responsible for the inability of the superinfecting virus to multiply and to cause cell injury.* This restriction does not invalidate the possibility, suggested by recent experimental evidence (Isaacs *et al.*, 1957; Isaacs and Lindenmann, 1957), that infected cells may release specific reaction products which, though not identifiable as virus in nature, may "transmit" interference to noninfected cells.

B. Criteria for Quantitative Analysis

The key to an understanding of the meaning and the mechanisms of interference lies in the fate of the interfering virus, of the suppressed virus, and in the specific changes induced in the infected cells by the interfering virus. Technically, interference is a manifestation of mixed infection of cells. This circumstance compounds the difficulties encountered in developing fruitful experimental approaches to the elucidation of the infectious process

itself. Until more is known about mechanisms of infection with single viruses, there is little hope of understanding the basis of interference. Nevertheless, in a purely technical sense, beginnings have been made in quantitative analysis of the following questions: (a) What is the least amount of interfering virus needed per cell? (b) How rapidly is interference established and how long does it last? (c) Is interference an all-or-none phenomenon, i.e., is an "interfered" cell entirely incapable of being superinfected or can the blocking effect be overcome? (d) Is partial interference due to incomplete inhibition of viral multiplication in all cells of a population or to reduced numbers of virus yielders? Most of the answers—as far as they go—have come from work on myxoviruses in the allantoic membrane and in tissue culture. They will be discussed in the appropriate sections of this chapter.

III. Experimental Systems

A. Demonstration of Interference in Mammalian and Avian Hosts

1. *Heterologous Viruses*

The broad spectrum of heterologous viral pairs and host systems with which interference of one sort or another has been demonstrated can best be appreciated by reviewing a summary, such as presented in Table I. Although most of our knowledge concerning quantitative aspects is derived from work on myxoviruses in chick embryos (see Section III, B) or on model systems in tissue cultures (see Section III, C), it would be misleading to disregard the mass of data available from studies in more complex hosts. It is here that the possible scope of the phenomenon will ultimately be related to the natural history of viral infections.

a. Criteria. The criteria used by different authors or by us in interpreting experimental findings as interference vary in reliability and rigidity. For critical analysis, it would be nice if one could limit discussion to instances which have been subjected to careful quantitation of *multiplication inhibition.* This cannot be, because much important information, especially about timing and dosage factors, has been obtained from studies in which interference manifested itself chiefly as *protection* against the pathological consequences of the challenge (suppressed) virus. The nature of such protection obviously depends on the host system, and may be against death or disease in animals, lesions, or cytopathogenic effects in tissue culture. It may be partial or complete, just as is inhibition of multiplication. Another criterion is the mutual *suppression of immunogenicity* found when two or more attenuated viruses are inoculated together (dengue-yellow fever). Of dubious validity is the gradual loss of one of a pair of viruses in the course of serial passages

TABLE I

INTERFERENCE BETWEEN HETEROLOGOUS VIRUSES

Interfering virus	Suppressed virus	Test system	Criterion	Reference
Influenza	Het. influenza	Ch. E. allantois	Multiplication inhibition	Henle and Henle, 1943, 1944*a*,b, 1945a,b; Ziegler *et al.*, 1944; Hirst, 1950; Isaacs and Edney, 1950, 1951; Fazekas de St. Groth and Edney, 1952; Fazekas de St. Groth *et al.*, 1952; Gotlieb and Hirst, 1954
Influenza	Het. influenza	Mouse lung	Multiplication inhibition	Ginsberg and Horsfall, 1949
Influenza	NDV	Ch. E. allantois	Multiplication inhibition	Isaacs and Edney, 1950; Hirst, 1950; Granoff and Hirst, 1954
Influenza	NDV	Ch. E. allantois	Protection	Florman, 1948; Bang, 1949
Influenza	Mumps	Ch. E. allantois	Multiplication inhibition	Ginsberg and Horsfall, 1949; Henle, 1950; Isaacs and Edney, 1950
Influenza	PVM	Mouse lung	Multiplication inhibition	Ginsberg and Horsfall, 1949
Influenza	EE	Ch. E. allantois	Protection	Henle and Henle, 1945a; Bang, 1949
Influenza	EE	Mouse CNS	Protection	Vilches and Hirst, 1947; Hirst, 1950
Influenza	EE	TC	Multiplication inhibition	Schlesinger, 1951; Levine, 1958
Influenza	EE	TC	Protection	Taylor, 1953
Influenza	SLE	Mouse CNS	Protection	Vilches and Hirst, 1947
Influenza	Bwamba	Mouse CNS	Protection	Vilches and Hirst, 1947
Influenza	West Nile	TC	Multiplication inhibition	Lennette and Koprowski, 1946
Influenza	Vaccinia	Ch. E. chorion	Protection and multiplication inhibition	Depoux and Isaacs, 1954a,b
NDV	Influenza	Ch. E. allantois	Multiplication inhibition	Florman, 1948
NDV	EE	Mouse CNS	Protection	Vilches and Hirst, 1947
NDV	EE	TC	Multiplication inhibition	Levine, 1958
NDV	VSV	TC	Protection and multiplication inhibition	G. Henle *et al.*, 1958

NDV	Polio	TC	Protection	Chanock, 1955
NDV	ECHO	TC	Protection	Chanock, 1955
NDV	Influenza	Ch. E. allantois	Multiplication inhibition	Granoff and Hirst, 1954
Mumps	Influenza	Ch. E. allantois	Multiplication inhibition	Ginsberg and Horsfall, 1949
Mumps	PVM	Mouse lung	Protection and multiplication inhibition	Ginsberg and Horsfall, 1951
Mumps	EE	Mouse CNS	Protection	Vilches and Hirst, 1947
PVM	Influenza	Mouse lung	Multiplication inhibition	Ginsberg and Horsfall, 1949
EE	Heterotypic EE	Mouse CNS, GP CNS	Protection	Schlesinger *et al.*, 1943, 1944
EE	VSV	Mouse CNS, GP CNS	Protection	Schlesinger *et al.*, 1943, 1944
EE	NDV	Chicken CNS	Protection	Bang, 1949
EE	NDV	TC	Multiplication inhibition	Levine, 1958
SLE	EE	TC	Protection	Huang, 1943
SLE	EE	Rats i.n.	Protection	Duffy *et al.*, 1952a; Duffy and Morgan, 1953; Jordan and Duffy, 1952
Jap B	EE	Rats i.n.	Protection	Duffy *et al.*, 1952b
Jap B	Polio	TC	Multiplication inhibition	Mason and Woodie, 1955
YF	RVF	Mouse	Protection	Findlay and MacCallum, 1937
YF	Dengue	Man	Protection	Sabin, 1952a
YF	Dengue	Man	Suppression of Ab form.	Schlesinger *et al.*, 1956
YF	West Nile	TC	Multiplication inhibition	Lennette and Koprowski, 1946
YF	EE (Ven.)	TC	Multiplication inhibition	Lennette and Koprowski, 1946
YF	EE (Ven.)	Ch. E.	Protection	Lennette and Koprowski, 1946
YF	Influenza	TC	Multiplication inhibition	Lennette and Koprowski, 1946
Dengue	Het. dengue	Man	Suppression of Ab form.	Schlesinger *et al.*, 1956
Dengue	YF	Man	Suppression of Ab form.	Schlesinger *et al.*, 1956
Dengue	YF	Mosquito	Multiplication inhibition	Sabin and Theiler (see Sabin, 1952b)
West Nile	EE (Ven.)	TC	Multiplication inhibition	Lennette and Koprowski, 1946
West Nile	Influenza	TC	Multiplication inhibition	Lennette and Koprowski, 1946
Polio	Het. polio	TC	Protection and multiplication inhibition	Ledinko and Melnick, 1954

TABLE I—INTERFERENCE BETWEEN HETEROLOGOUS VIRUSES (*cont.*)

Interfering virus	Suppressed virus	Test system	Criterion	Reference
Polio	Het. polio	TC	Protection	LeBouvier, 1954; Sabin *et al.*, 1954 Drake, 1958
Polio	Coxsackie	TC	Protection	LeBouvier, 1954
Polio	EMC	Hamster CNS	Protection	Dalldorf and Whitney, 1943
Coxsackie	Polio	Mouse i.p.	Protection	Dalldorf, 1951
Coxsackie	Polio	Mouse CNS	Protection	Stanley, 1952; Sulkin *et al.*, 1953
Theiler	EE	Mouse CNS	Protection and multiplication inhibition	Schlesinger *et al.*, 1943
EMC	Polio	Monkey CNS	Protection	Jungeblut and Sanders, 1942
EMC	EE	TC	Protection	Huang, 1943
LCM	Polio	Monkey CNS	Protection and multiplication inhibition	Dalldorf *et al.*, 1937, 1938; Dalldorf, 1939; Dalldorf and Douglass, 1938
VSV	Het. VSV	TC	Multiplication inhibition	Cooper, 1958
Fowl Pox	Vaccinia	Ch. E. CAM	Histology	Anderson, 1942
Vaccinia	F and M	GP skin	Protection	Gildemeister and Helm, 1932
Psitt.-lgr. ven.	Het. psitt.-lgr.	Mice i.p.	Multiplication inhibition	Golub and Wagner, 1948a
Psitt.-lgr. ven.	Het. psitt.-lgr.	Ch. E. yolk sac	Multiplication inhibition	Golub and Wagner, 1948b
Papilloma (Shope)	Sheep dermatitis	Rabbit skin	Protection	Selbie, 1946
dba mammary Ca (?)	Vaccinia	Ch. E. CAM	Protection	Taylor and Carmichael, 1953

Abbreviations:

Het: heterotypic.
Ch.E.: chick embryo; TC—tissue culture; GP—guinea pig.
NDV: Newcastle disease virus.
PVM: pneumonia virus of mice.
EE: equine encephalomyelitis (WEE, EEE, or Venezuelan EE).
SLE: St. Louis encephalitis.
VSV: vesicular stomatitis virus.
Jap B: Japanese type B encephalitis.
YF: yellow fever.
RVF: Rift Valley fever.
EMC: encephalomyocarditis.
LCM: lymphocytic choriomeningitis.
F and M: foot-and-mouth disease.
Psitt.-lgr.: psittacosis-lymphogramuloma venereum group.
i.n. intranasal.
i.p.: intraperitoneal.
i.m.: intramuscular.
s.c.: subcutaneous.

initiated with mixedly infected organs (Levaditi, 1942 a,b,c, 1943; Levaditi and Henry-Eveno, 1952; Levaditi and Noury, 1943 a,b,c, 1944a,b; Levaditi and Reinié, 1940, 1941; Levaditi and Vaisman, 1951a,b,c,d,e; Levaditi *et al.*, 1952a,b). In absence of any quantitative evaluation, especially with regard to relative rates of multiplication of the juxtaposed viruses, it is impossible to relate these observations to interference.

b. Independence of Antigenic Relationship and Antibody Production: Cross Resistance and "Minor" Group Antigens. Many of the viral pairs listed in Table I testify to the fact that interference can occur between antigenically and taxonomically unrelated viruses. This point requires no further comment. Moreover, interference in embryonated eggs or in tissue culture obviously does not involve antibody production. A more subtle difficulty arises in assessing the possible role of interference as a mechanism underlying induction in animals of reciprocal resistance to viruses which share only some antigenic properties. Specifically, what is the significance of antibodies to "minor" group antigens in the evolution of resistance to heterologous viruses of a single group? For example, past exposure to one type of poliovirus so sensitizes monkeys or human beings that vaccination with a different type induces production of neutralizing antibody against all three types (Salk, 1956). Similarly, consecutive infection with different members of the arthropod-borne viruses of group B which share CF and HA antigens (Sabin, 1950; Sweet and Sabin, 1954; Casals and Brown, 1954) induces formation of broadly cross-reacting neutralizing antibodies (Smithburn, 1954; Schlesinger *et al.*, 1956; Imam and Hammon, 1957).

In terms of resistance to reinfection, it is striking that monkeys *vaccinated* with inactivated polioviruses were found protected against intracerebral challenge doses of only homotypic virus (Morgan, 1949). In contrast, when polio-*convalescent* monkeys were reinoculated intracerebrally with heterotypic strains, a considerable proportion was found resistant (Bodian, 1949). Bodian observed not only an inverse relationship between the severity of primary paralysis and frequency of successful heterologous reinfection, but also a localization of protection to previously involved extremities. He rejected interference as a basis of cross resistance because it persisted for up to 13 to 22 weeks, i.e., longer than virus was then assumed to remain associated with cells of the CNS. Since then, Bodian (1957) has shown that poliovirus in demonstrable concentrations does persist in the spinal cord of paralyzed monkeys for 4 weeks. This finding, together with the demonstration of interference between heterotypic polioviruses in tissue culture (see Table I), offers a stronger case for the previously rejected interpretation.

The situation may be similar in the case of types 1 and 2 dengue virus in man. Sabin (1952b) found that human volunteers convalescent from infection with one type acquired evanescent resistance (for about two to six months) to

challenge with a heterotypic strain, while homologous resistance lasted for at least two years. It seems quite reasonable to suggest that early protection against heterologous strains may have been due to interference rather than to cross-reacting antibody.

The distinction between specific immunity and interference as the basis for host resistance was brought out sharply in studies on eastern and western equine encephalomyelitis (EEE and WEE) viruses by Schlesinger *et al.* (1943, 1944). Vaccination of rabbits, guinea pigs, or mice with formalin-killed WEE virus induced long-lasting protection against intracerebral challenge doses of the homologous, not the heterologous virus. Homologous challenge led to transient infection whose abortion was associated with a marked local, type-specific antibody response (Schlesinger *et al.*, 1944; Schlesinger, 1949). After such an abortive infection, the animals resisted intracerebral superinfection with massive doses of the heterologous virus. This type of resistance was effective for relatively short periods of time and was not associated with anamnestic antibody response. It was therefore considered as due to interference.

Influenza has provided experience on a much larger scale than the examples cited to show that "minor" cross-reacting antigens and antibodies do not afford protection against variant strains, even of a single serotype. Evidence is rapidly accumulating which points to the persistent association of viruses with cells—both in tissue cultures and in the intact host. Indeed, presence of protective antibody encourages rather than discourages lingering infection (Ackermann and Kurtz, 1955). It seems reasonable to propose that such conditions may set the stage for effective maintenance of interference as a mechanism of cross protection. Apparently a curious paradox has arisen in our thinking about this phenomenon: because the most penetrating studies on interference have been done on simple model systems, viz., the allantoic membrane and tissue cultures, we tend to shrug off the possibility that it may be an inevitable and significant phase in the evolution of any, even the natural, host-virus relationship.

2. *Homologous Viruses*

In general, the demonstration of homologous interference depends on a difference in virulence between two variants of the same virus. The evaluation of such systems is based either (a) on protection of the host (or of cells) against the virulent variant or (b) on assay of the viral progeny for the virulent component. Examples of this sort, other than those concerned with influenza virus in eggs, are listed in Table II. The interfering agent in all these cases is *active* virus. Since interfering and challenge virus are antigenically indistinguishable and genetically related, the distinction of interference from immunological phenomena and genetic interaction is much more difficult than for heterologous pairs.

TABLE II

INTERFERENCE BETWEEN HOMOLOGOUS VIRUSES [a]

Virus	Interfering variant	Suppressed variant	Test system	Criterion	Reference
YF	Neurotropic	Viscerotropic	Monkey s.c. or i.p.	Protection	Hoskins, 1935; Findlay and MacCallum, 1937
YF	17DD (TC)	Asibi monkey	TC	Multiplication inhibition	Lennette and Koprowski, 1946
Herpes simplex	Nonencephalitogenic	Encephalitogenic	Rabbit CNS	Protection	Magrassi, 1935; Doerr and Kon, 1937; Doerr and Seidenberg, 1937; Hallauer, 1937
Influenza	Egg-adapted WS	Neuro-WS	TC	Multiplication inhibition	Andrewes, 1942
Theiler	Low virulence	High virulence	Mouse CNS	Protection and multiplication inhibition	Gard, 1944
Vaccinia	Dermotropic	Neurotropic	Mouse CNS	Protection	Dalldorf *et al.*, 1947
Distemper	Ferret-adapted	Virulent	Fox i.n. or i.m.	Protection	Green and Stulberg, 1946
Distemper	Egg-adapted	Ferret-adapted	Ferret	Protection	Schindler, 1955
Rinderpest	Egg-adapted	Virulent	Calf	Protection	Jenkins and Shope, 1946
NDV	Avirulent	Virulent	Chicken	Protection	Bang, 1949; Russeff, 1955
Dengue	Avirulent	Neurotropic	Mouse CNS	Protection	Schlesinger and Frankel, 1952b
LCM	Nonviscerotropic	Viscerotropic	Mouse i.p.	Protection	Rowe, 1954
Polio	Noncytopathogenic	Cytopathogenic	TC	Protection	Sabin, 1954; LeBouvier, 1954

[a] Not including myxoviruses in eggs.

Abbreviations:

TC—tissue culture.
NDV: Newcastle disease virus.
YF: yellow fever.
LCM: lymphocytic choriomeningitis.
i.n.: intranasal.
i.p.: intraperitoneal.
i.m.: intramuscular.
s.c. subcutaneous.

a. Distinction from Immunological Phenomena. In the classic studies by Hoskins (1935) and by Findlay and MacCallum (1937) on interference by neurotropic with viscerotropic yellow fever virus, a major element in ruling out immunological mechanisms was the cross-protection, achieved in similar manner, between yellow fever and Rift Valley fever (RVF) viruses. In other cases, e.g., in the "Konkurrenz-phänomen" of Magrassi (1935) between non-encephalitogenic and encephalitogenic strains of herpes virus and the subsequent demonstration for the same system of "Schienenimmunität" along neuronal pathways (Hallauer, 1937; Doerr and Kon, 1937; Doerr and Seidenberg, 1937), the possible role of antibody cannot be denied. The time interval between "interfering" and challenge virus was long enough to permit sensitization of the antibody-producing cells by the primary inoculum. Thus, it is conceivable that the challenge dose stimulated a secondary antibody response of the kind demonstrated in the brain of WEE-vaccinated animals after intracerebral challenge with active WEE virus (Schlesinger, 1949). In other words, absence of antibody at the time of challenge inoculation does not necessarily mean that the challenge virus may not itself serve as an antigenic booster dose adequate to ensure effective protection. The possible interplay of interference and specific immune mechanisms is illustrated, for example, by the work of Karzon and Bang (1951) on avirulent (B) and virulent (CG 179) strains of Newcastle disease virus (NDV) in chickens, which suggests a connection between local antigenic booster effect and resistance to the virulent variant. In a previous communication (Bang, 1949), this type of protection had been considered as due to "cell blockade" or interference. Similar relationships may be involved in Gard's (1944) studies on mixed infection with avirulent and virulent strains of Theiler virus. This question has to be settled for each system separately and fortunately does not obscure the situations involving chick embryos or tissue cultures.

b. "Autointerference"—Its Relation to Heterogeneity of Viral Populations and Genetic Interaction. Autointerference expresses itself in terms of protection or of reduced viral multiplication in hosts inoculated with large doses of a virus which, in smaller doses, is pathogenic and multiplies to high levels. Pasteur's report on rabies in rabbits (1888): ". . . La rage a paru se déclarer à la suite d'un quart de seringue plus fréquemment que par une ou plusieur seringues . . ." states in principle the situation for the following systems: influenza B in chick embryos (Groupé and Pugh, 1950); yellow fever (Smithburn, 1949; Theiler, 1951); dengue (Schlesinger and Frankel, 1952a; Smith, 1956); and RVF (Mims, 1956) in mice; plaque production by egg-adapted influenza strains on chick embryo lung monolayers (Granoff, 1955a); WEE virus in L cells (Chambers, 1957); vesicular stomatitis virus (VSV) in chick embryo monolayers (Cooper, 1958). "Autointerference" obtained with influenza

viruses in the allantoic cavity and leading to production of "incomplete" virus will be discussed separately (Section III, B, 4).

Superficial analogies have led some authors to postulate that other types of autointerference are likewise associated with "incomplete" virus production. For example, Mims (1956) found that serum of mice inoculated with undiluted RVF virus often contained virus of low infectious titer but of antibody-combining capacity equivalent to that of high-titer serum. Quite apart from reservations one may have about the relation of interference to the genesis of "incomplete" influenza virus (see Section III, B, 4), it seems noteworthy that autointerference with other systems is characteristic especially of viruses which are not fully adapted to the experimental host or which are mixtures of particles with different properties (in Mims' experiments, e.g., viscerotropic and neurotropic RVF particles). The inoculation of such viral populations could be akin to that of artificial mixtures of avirulent and virulent particles resulting in (a) suppression of multiplication of the virulent component and (b) protection of the host. To determine the presence of avirulent rather than "incomplete" progeny would then require an indicator host susceptible to the avirulent component.

This approach has been possible in an investigation of "autointerference" with a strain of Type 2 dengue virus in adult mice (Schlesinger and Frankel, 1952a,b; Schlesinger *et al.*, 1958). This strain was adapted to suckling mice by intracerebral passages, and after 4–6 passages its lethal titer became fixed around 10^{-7}. Additional serial passages in sucklings brought about a gradual increase in virulence for young adult mice. At low passage levels, titration of the virus by intracerebral inoculation into adults often resulted in autointerference, i.e., highest mortality rates among those inoculated with high dilutions of virus. Transfer back to sucklings, as indicator hosts for virus lacking adult virulence, revealed that such virus multiplied in the brains of adult mice even when it produced no illness in them. Had such an indicator host not been available, one might have thought that "incomplete" virus was involved.

Moreover, after some 25 passages in sucklings, the virus attained the same lethal titer for adults as it had for sucklings. Titrations of such high passage virus never gave a pattern of autointerference. When adult mice were doubly infected with low- and high-passage virus, protection against the latter was observed. The relative effectiveness of protection was proportional to the timing or dosage advantage of the avirulent component. There was, however, one significant exception: Mixtures containing sublethal (0.1 to 0.01 LD_{50}) amounts of the virulent and 1 to 10^3 LD_{50} (titer in suckling mice) of the avirulent virus induced mortality rates in excess of the combined rates for the two components given singly. How could this paradox be explained? By analogy to the experiments on reactivation of partially inactivated

influenza virus by double infection of cells with active virus (see below), it might be proposed that sublethal concentrations of the virulent virus contained some particles by themselves incapable of initiating fatal infection. If cells infected with such particles were superinfected with partially adapted particles, recombination might lead to emergence of virulent progeny. Such progeny could conceivably overgrow the avirulent component since the latter was given in relatively low concentration. The system under study unfortunately did not lend itself to a critical test of this hypothesis. Whatever the mechanism of this strange potentiation, its occurrence may be relevant to that of autointerference itself. In the case of influenza viruses, the idea of intrinsic heterogeneity of viral populations has much experimental support (see Section III, B, 4), and the extent to which interference and genetic interactions are interwoven can be amply documented. In other cases, where virulence or lack of virulence are the only distinguishing markers, it may be impossible to choose between the two alternatives.

3. *Interfering Capacity of Inactivated Viruses*

Some inactivated viruses with which interference of one sort or another has been induced are listed in Table III. The vast amount of work done with inactivated myxoviruses will be taken up in detail in Section III, B. The other systems listed have contributed little to our basic understanding of interference other than by confirming that infectiousness, i.e., demonstrable ability to multiply, is not a prerequisite in all systems.

4. *The Role of Timing and Dosage*

Aside from the work with myxoviruses in eggs and tissue culture (see below), relatively few systems have been subjected to careful quantitative analysis. In all systems utilizing inactivated viruses as interfering agents, their effectiveness depends on administration of saturating amounts. When active virus is used, the amount needed to induce interference varies with its capacity to multiply in the host system and on its rate of multiplication relative to that of the suppressed virus. A good example illustrating these points is the interference by egg-adapted influenza strains with WEE virus in brains of mice (Vilches and Hirst, 1947). When the two viruses were given simultaneously, interference was induced by $10^{4.9}$ but not by $10^{4.2}$ EID_{50} of the WS strain, and the amount of WEE virus required to overcome this effect increased with greater concentration of WS virus. The infectious titer of the interfering agent in mouse brain dropped progressively from the time of inoculation until none was recovered after 5 days. Despite this limited activity of the interfering virus, interference by large doses was demonstrable for up to 15 days, though decreasing in effectiveness from the seventh day on. Several points of interest emerge from Vilches and Hirst's study: (a) the

TABLE III

INTERFERENCE BY INACTIVATED VIRUSES [a]

Interfering virus	Method of inactivation	Suppressed virus	Test system	Reference
Ectromelia	UV	Ectromelia	Mouse	Andrewes and Elford, 1947
Psitt.-lgr.	UV or heat	Psitt.-lgr.	Ch. E. YS	Golub and Wagner, 1948b
Infectious bronchitis	Heat	Infectious bronchitis	Ch. E. allantois	Groupé, 1949
Vaccinia	UV	Vaccinia	Rabbit cornea	Nagano and Furuno, 1951
Vaccinia	UV	Vaccinia	Rabbit skin	Nagano *et al.*, 1954
Vaccinia	UV	Vaccinia	Ch. E. CAM	Matumoto and Shinkawa, 1956
RVF	UV	RVF	Mouse	Naudé and Polson, 1957

[a] Other than myxoviruses in eggs or tissue culture.

Abbreviations:

Ch.E.: chick embryo.
Psitt.-lgr.: psittacosis-lymphogranuloma venereum group.
RVF: Rift Valley fever.
UV: ultraviolet.
YS: Yolk sac.

demonstration of gradual "overcoming of interference" with increasing intervals between the two inoculations; (b) the fact that suppression of a slowly multiplying variant (Lederle) of WEE virus was much more effective than of a rapidly multiplying one (Rockefeller Institute strain). This aspect has gained added significance as a result of studies by Levine (1958), suggesting that NDV and WEE virus are mutually competitive in tissue culture (see Section III, C); (c) in keeping with the restricted multiplication of influenza virus in mouse brain (Schlesinger, 1950, 1953; Cairns, 1951, 1954), there was no significant increase in the amount of WEE virus suppressed as the interval between the two inoculations was lengthened from 0 to 7 days. This is contrary to systems in which the interfering virus multiplies and spreads from cell to cell, as exemplified by the inhibition of WEE virus in mice previously inoculated intracerebrally with the TO strain of Theiler virus: the longer the interval between the two inoculations, the greater the resistance to WEE virus (Schlesinger *et al.*, 1943; Schlesinger, 1952).

Although conclusions based on experiments done on intact animals are in general agreement with the principles governing interference in the allantoic cavity or in tissue culture, they do not lend themselves to truly quantitative interpretations.

5. *Localized Character*

Interference is set apart from systemic immune mechanisms not only by specialized requirements with regard to timing and dosage but also by its localized character. Thus, localization of protection to neuronal pathways traveled by interfering virus was demonstrated by Magrassi (1935) and by Doerr *et al.* (Doerr and Kon, 1937; Doerr and Seidenberg, 1937) and Hallauer (1937) with herpesvirus, and later by Jordan and Duffy (1952) for heterologous pairs, such as SLE and WEE viruses. The work by Sulkin *et al.* (1953) also suggests that pre-emption of certain levels of the spinal cord of mice by Coxsackie virus may afford local protection against poliovirus. Henle *et al.* report (1947a) that the amniotic sac of chick embryos whose allantoic membrane is blocked by UV virus retains full susceptibility to active influenza virus. Henle and Henle (1945a) also have shown that inactivated influenza virus protects chick embryos against death due to WEE virus, provided great care is taken to ensure that the latter has primary access only to allantoic cells. When such precautions are not taken, no protection is achieved (Vilches and Hirst, 1947). Similarly, Bang (1949) reports protection by inactivated against active NDV when both are inoculated on the dropped CAM, but not when the active challenge virus is inoculated intra-allantoically. The situations involving protection against lethal effects of myxoviruses are complicated by the possible role of "toxic" factors and by variations in the ease with which different strains spread systemically to the embryo, which

may explain in part seemingly conflicting reports (e.g., Isaacs and Edney, 1950a; Burnet and Fraser, 1952). At any rate, the overwhelming independent evidence pointing to the cellular mechanism of interference would be incompatible with the idea that protection of cells or organ systems at a distance from those directly exposed to the interfering agent might be due to interference.

B. Interference among Members of the Myxovirus Group in the Allantoic Membrane of Chick Embryos (CAM)

Since the original demonstration of reciprocal interference between type A and type B strains of influenza virus (Henle and Henle, 1943; Ziegler *et al.*, 1944), the system, with various modifications, has revealed the complex way in which interference and genetic interactions between viral particles are interwoven. This is true particularly for experiments with antigenically related strains of virus.

1. *Heterology and Homology Among Myxoviruses*

The six members of the myxovirus group (*Myxovirus influenzae A, influenzae B, influenzae C, multiforme, pestis galli, parotitidis*) are considered as heterologous with respect to one another (Andrewes *et al.*, 1955). At the other extreme, two laboratory-bred variants of a single strain, e.g., egg-adapted and neurotropic WS, are obviously homologous. A special problem arises with different strains of, say, influenza type A, which, though sharing antigenic constituents, differ by strain-specific antigens. Are these strains less different from one another than, for example, the different members of group B arborviruses? At least we can infer evolutionary relatedness of type A influenza strains from the historical evidence of a continuous "immunological drift" (Burnet, 1955) which imprints new antigenic specificities on newly arising strains (Hirst, 1952). This homology is borne out by results of recombination experiments suggesting that exchange of genetic materials occurs not only with laboratory-induced variants of a single strain (e.g., CAM: Burnet and Lind, 1954d) but also with different strains, be they historically close (WS and MEL: Burnet and Edney, 1951; Burnet and Fraser, 1952; Burnet and Lind, 1951a,b, 1952, 1954a,b,c; Hirst and Gotlieb, 1953a,b, 1955; Lind and Burnet, 1953-1957; Gotlieb and Hirst, 1954, 1956) or far apart (WS and FM1, Hirst and Gotlieb, 1953a; WS and CAM, Burnet and Lind, 1955; WS and various strains, Burnet and Lind, 1956). Assuming that mixed infection with all these pairs may give rise to diploid or heterozygous particles comparable to those postulated for the WS-MEL system (Gotlieb and Hirst, 1954) or to truly homozygous recombinants (Lind and Burnet, 1957a), and that, contrariwise, progeny from mixed type A-type B infection or from influenza-NDV infection contains "phenotypically mixed," but not genetically mixed,

particles (Gotlieb and Hirst, 1954; Granoff and Hirst, 1954), a criterion for designating all A strains (or all B strains) as homologous in relation to one another would be established. Although this assumption is premature, adherence to it facilitates logical sequence of discussion.

2. *Interference and Mixed Infection with Two Active Myxoviruses*

a. Heterologous Systems. The observation that infection with a type A strain (PR8) could lead to suppression of superinfecting type B (LEE) virus, and vice versa, was first reported by Ziegler and Horsfall (1944). They showed that (a) both viruses could multiply side by side if small amounts of each were inoculated; (b) the direction and efficacy of interference depended on the temporal and dosage advantage of one over the other strain. Other conditions being comparable, PR8 was more effective as interfering agent than LEE, and this was in line with its more rapid rate of multiplication.

Because of the dominant interest then commanded by work on interference by inactivated influenza virus, not much more was done on this problem. It was tacitly assumed that a cell infected by PR8 virus could not support multiplication of LEE and vice versa, and that mixed yields indicated that the initial infecting dose had not sufficed to infect all cells with both viruses.

This all-or-none concept of mutual exclusion was shattered when Sugg and Magill (1948), Sugg (1951), and Liu and Henle (1951b) found that, under carefully controlled conditions, mixed PR8-LEE infection could be propagated serially through as many as 52 passages. Moreover, Liu and Henle (1953) found that subpassage in limiting dilution (1/2–1/8 ID_{50}) of virus harvested from mixedly infected eggs often yielded virus of both serotypes. Further analysis of the phenomenon by Gotlieb and Hirst (1954) suggested that a single doubly-antigenic particle, obtained from mixedly infected eggs, could give rise to either one or the other serotype, not to both. This led them to postulate that such particles were "phenotypically mixed." The same situation was found for the system MEL-NDV (Granoff and Hirst, 1954). The occurrence of phenotypically mixed particles can be explained only if one assumes that A and B or influenza and NDV particles *can* infect a single cell and that mutual exclusion therefore is not an all-or-none effect. By defining the relative concentration of each component in mixtures optimal for obtaining such doubly antigenic progeny, Hirst and co-workers at the same time crystallized more sharply the conditions for mutual interference. The relative proportions favoring release of doubly antigenic progeny were different for the two systems, but in both small shifts in either direction led to preponderance in the yield of either one or the other component virus. It should be noted that the PR8-LEE mixtures at ratios 2 : 1 to 8 : 1 yielded predominantly LEE, in contrast to the earlier findings by Ziegler and Horsfall (1944) with smaller infecting doses. This may be related to the fact that the constant

period preceding viral increase is shortened in eggs infected with large doses of LEE (Liu and Henle, 1951b).

Other systems involving interference between active myxoviruses in the allantoic cavity (see Table I) must be interpreted in the light of these subtle quantitative requirements. This applies particularly to the apparent conflict concerning interference between influenza and mumps viruses. Ginsberg and Horsfall (1949) claimed that these two agents were not mutually exclusive. Actually their experiments showed significant reciprocal suppression of multiplication, an inference borne out by subsequent work by Isaacs and Edney (1950a) and by the Henles (see Henle, 1950).

b. Homologous Systems. The interplay of interference and genetic interaction in mixed infection with two homotypic strains of influenza virus differing in strain-specific antigens or virulence and other markers was first demonstrated by Burnet and Edney (1951) and Burnet and Lind (1951a,b) with MEL and NWS in mouse brain. In mixed infections of eggs, the quantitative principle was similar to that described for heterologous pairs in that distinct advantage of one over the other strain resulted in suppression of the latter. When both were given in dynamically equivalent amounts, the yield contained a high proportion of recombinant particles (Hirst and Gotlieb, 1953a). In contrast to the situation for heterologous pairs, Gotlieb and Hirst (1954) have offered evidence suggesting that the genetic compatibility of homologous pairs, e.g., MEL and WS, expresses itself in that recombinants are not merely phenotypically mixed but may behave as heterozygous or diploid particles as well. Are the methods commonly used for detecting phenotypic or genetic recombinants sufficiently sensitive to reveal their presence under unbalanced circumstances, where one strain has a decided quantitative advantage over the other? In other words, is the phenotypic suppression of one strain under such circumstances really an expression of interference in the strict sense (mutual exclusion) or of decided genetic or phenotypic dominance of one parent type in recombinant progeny? The evidence at hand does not provide an answer.

3. *Interference and Genetic Interaction between Inactivated and Active Myxoviruses*

The interfering capacity of influenza viruses is retained after partial or complete abolition of infectivity by UV-irradiation (Henle and Henle, 1943; Ziegler *et al.*, 1944; Powell and Setlow, 1956), ionizing radiation (Powell and Pollard, 1956), heat or formaldehyde (Henle and Henle, 1943, 1944a; Isaacs and Edney, 1950a), or sulfur mustard (Fong and Louie, 1953). Earlier data by Henle and Henle (1947) on the UV-sensitivity of the interfering component of the viral particle have been supplemented by studies of Powell *et al.* (Powell and Pollard, 1956; Powell and Setlow, 1956) on sensitivity to ionizing

and monochromatic UV-radiation. The active site responsible for interference appears to reside in a minute fraction of the viral particle (2×10^6 Å^3 volume, mol. wt. 1.6×10^6). The action spectrum of UV-inactivation suggests that the active moiety is protein in nature, and Powell and Setlow feel that it might be associated with viral RNA but that a possible role of lipids cannot be ruled out.

Until very recently, all experimental evidence pointed to the inseparable identity of the interfering agent with the physical entity of the inactivated viral particle (Henle and Henle, 1945b; Ziegler *et al.*, 1944; Isaacs and Edney, 1950a; Fazekas de St. Groth and Edney, 1952). Paucker and Henle (1958) have found that neither the HA component nor the S antigen obtained from elementary bodies by treatment with ether have any interfering capacity. The possible role of a nonviral macromolecular material ("interferon") liberated by allantoic cells after interaction with inactivated virus (Isaacs *et al.*, 1957; Isaacs and Lindenmann, 1957; Lindenmann *et al.*, 1957) and capable of passively transmitting refractoriness to uninfected cells will be discussed below.

a. Heterologous Systems. The original observations by Ziegler *et al.*, (1944) and the Henles (1944b, 1945a) were compatible with the view that exposure of the allantois to UV-virus at concentrations sufficient to ensure "infection" of each cell resulted in complete immediate blockade of the cells to superinfecting, heterologous active virus. Fazekas de St. Groth and Edney (1952) calculated that a single particle of inactive virus per cell completely prevented the multiplication in that cell of active heterologous virus added after an interval of 24 hours. Subsequently, Fazekas de St. Groth and associates (1952) demonstrated that in eggs inoculated first with a large dose of heated LEE and 24 hours later with 100 ID_{50} of MEL the latter multiplied, but at a rate much lower than in control eggs. Nevertheless, the final titer which the virus attained after about 50 hours was only 1.15 $\log_{10}$ units lower than the maximum yield in control eggs reached at about 30 hours. When the interval between the two inocula was shortened to 45 minutes, the growth rate of MEL was initially as in control eggs. It changed from the fifteenth hour onward (after about two cycles) to the same slope and ended at the same final yield as in the 24-hour series. It was concluded that interference was not established during the first few hours after inoculation of heated virus. In the light of the important observation of Finter *et al.* (1954) that allantoic cells, once infected, continue liberating virus at a constant rate for some 36 hours, the data of Fazekas de St. Groth *et al.* indicate that a proportion of cells (about 7 %) escaped interference even after 24 hours and liberated virus in normal fashion. Along similar lines, Henle and Paucker (1958) found that saturating doses of interfering virus led to *complete* interference after 9 to more than 24 hours, depending on the multiplicity of adsorption of inactive virus. Up to this

time, the yield of newly produced virus was directly proportional to the amount of challenge virus inoculated but gradually decreasing with increasing time intervals. The fact that these authors, in contrast to Fazekas de St. Groth, obtained no evidence suggesting that some cells escaped the interfering effect altogether may be due to the greater efficiency of UV-irradiated as compared with heated virus (Henle, personal comment).

It can be concluded that (a) a single inactivated viral particle can induce interference in a cell; (b) establishment of complete inhibition requires about 16 to 24 hours of contact between inactivated virus and cell; (c) a proportion of exposed cells may escape the interfering effect of the inactivated virus; (d) the yield of new virus from "interfered" cells decreases progressively until complete inhibition is established.

b. Homologous Systems. The marked depressing effect of inactivated influenza virus on the yield of superinfecting, homologous, active virus was demonstrated by Henle and Henle (1943, 1944a,b), Ziegler *et al.* (1944), and Isaacs and Edney (1950, 1951). They showed that inoculation of heat-or UV-inactivated virus in high concentration resulted in relatively low yields in terms of both infectious and HA titers. The conditions under which these observations were made closely paralleled those already described for heterologous interference. The first inkling of a fundamental difference between homologous and heterologous interference came with the discovery by Henle *et al.* (1947b) and Henle and Rosenberg (1949) of the effect of UV-irradiated virus inoculated *after* infection with active virus. They found that *heterologous* UV-virus did not affect multiplication in already infected cells; it did, however, block previously uninfected cells and thus viral multiplication was limited to a single step. *Homologous* UV-virus, in contrast, blocked not only uninfected cells but also suppressed production of infectious virus in the cells infected by the primary inoculum. The completeness of this block depended upon (a) the time interval (complete at 1 hour, decreasing progressively to the sixth hour), (b) the dosage. Active PR8 was effectively inhibited by inactive PR8, MEL, WS, and partially by Swine. The contrast here between homologous and heterologous pairs suggested that a cell already engaged in synthesis of viral material could still accept additional homologous virus. These earlier results of Henle *et al.* were based on infectivity titrations of the progeny only. Later findings (Liu *et al.*, 1956) suggested that superinfection with homologous, irradiated virus may actually result in the production of "incomplete" virus rather than in total suppression of viral replication.

When inactive and active homologous virus are inoculated either simultaneously or consecutively in the order named, additional evidence for multiple infection of single cells is obtained. Henle and Liu (1951) reported that multiplicity reactivation occurred in eggs inoculated with large doses of

UV-virus. Subsequently, it was shown by Burnet and Lind (1954a), and by Baron and Jensen (1955), and Gotlieb and Hirst (1956), through use of inactivated and active viruses with distinct genetic markers, that reactivation did indeed occur and that the inactivated parent contributed markers to combinant progeny particles. Of special interest in connection with the problem of interference is the observation by Gotlieb and Hirst (1956) that such interaction occurred even when the interval between inoculations of inactivated (UV-M−) and active (W^+) virus was extended to 16 hours. Moreover, the yield of recombinants under these conditions was surprisingly high.

Burnet (personal communication) finds that under similar conditions the effect depends on whether heated M^+ and active WS^- (recombination, no interference) or heated WS^- and active M^+ (interference, no recombination) are used. This suggests to him utilization of different processes for the two phenomena. It is clear that critical distinction between partial interference and recombination in homologous systems can be made only through genetic analysis of the viral yield.

4. *Autointerference and the Genesis of "Incomplete" Influenza Virus*

The subject of incomplete virus production is fully discussed in Chapter VI. The phenomenon requires comment here because it has been interpreted as a manifestation of autointerference in the sense that simultaneous infection of a cell with infectious and noninfectious particles leads to liberation of immature particles (von Magnus, 1951a,b, 1952). This concept presupposes (a) that the liberated noninfectious HA ("incomplete" virus) is the equivalent of a normal development stage in the genesis of infectious virus; (b) that its premature release depends on multiplicity of infection of single cells; (c) that interference here takes the form of arrested viral development, not of mutual exclusion.

Several cogent reasons have been cited for looking upon the genesis of incomplete virus in different light. Cairns and Edney (1952) suggested that multiplicity of infection is not required to obtain a high proportion of noninfectious virus in first-cycle yields. Burnet *et al.* (1954) were led to conclude that there were various degrees of incompleteness and that some forms were capable of limited reproduction. Henle and associates (Finter *et al.*, 1955; Paucker and Henle, 1955a,b; Liu *et al.*, 1956), working with partially inactivated virus as seed inoculum, also offered evidence supporting the view that cells infected only with inactivated viral particles yielded newly produced noninfectious hemagglutinin. One may add to these points the following considerations: (1) strain-specific variations in the ease with which incomplete virus can be obtained in eggs (von Magnus, 1954; Fazekas de St. Groth and Graham, 1954); (2) the restriction of multiplication of some viruses

in certain host systems to production of noninfectious HA and CF antigen (Schlesinger, 1950, 1953; Cairns, 1951, 1954; Fulton and Isaacs, 1953; Ginsberg, 1954; G. Henle *et al.*, 1955); (3) the demonstration that small noninfectious HA particles can be isolated from the infected CAM even under conditions where the liberated yield consists of "standard" virus: these small tissue-bound particles differ in physical and biological properties from incomplete virus obtained on serial undiluted passage (Granoff, 1955b) and are probably truly representative of a developmental phase of the virus (Henle *et al.*, 1956); (4) incomplete virus may contribute genetic markers to recombinants produced in de-embryonated eggs doubly infected with active virus (Burnet *et al.*, 1954).

It is known that even standard preparations of virus contain varying amounts of noninfectious HA (Gard *et al.*, 1952). As suggested previously (Schlesinger, 1953), production or accumulation of noninfectious virus may then be expected to occur under conditions favoring abnormal or functionally deficient particles which are present to begin with in a heterogeneous viral seed. We face the dilemma that our definition of infectiousness of influenza virus is synonymous with demonstrable production of new extracellular virus. "If the basis for this idea were applied to bacterial viruses, a temperate particle would be labelled 'incomplete' because it fails to give rise, in the majority of infected cells, to (extracellular) progeny" (Schlesinger, 1953). This viewpoint has been expanded by Burnet (1955, p. 141): ". . . every bacterial population contains an enormous variety of genetically incomplete forms, the great majority of which, being at a disadvantage for survival, give rise to no descendants. The situation is probably analogous for the replication of all viruses. In addition to the standard form, we have a variety of genetically imperfect forms. Only in the case of viruses like influenza, where we have a means of titrating the whole population (the HA titer), is it possible to provide an estimate of the proportions showing various grades of imperfection . . .". If this postulated intrinsic heterogeneity of viral populations is at the root of incomplete virus production, then interference is related to it only insofar as the genetically imperfect forms have a quantitative advantage over the standard forms.

5. *Interfering Capacity of "Incomplete" Virus*

Aside from the evidence derived from undiluted passage series in eggs (von Magnus, 1951a,b, 1952, 1954; Fazekas de St. Groth and Graham, 1954), other independent studies have shown that "incomplete" virus can interfere with infectious homologous or heterologous influenza virus. Burnet *et al.* (1954) have shown this in experiments in de-embryonated eggs. Powell and Pollard (1956) have reported that the interfering capacities of incomplete and artificially inactivated virus are equivalent. Suitable quantitative

conditions are given whenever a saturating seed inoculum contains a preponderance of noninfectious virus. As with active or artificially inactivated virus, effective blockade at the cellular level is not established until several hours after exposure (Burnet *et al.*, 1954). The existence of different kinds of "incomplete" virus is borne out by the finding of a correlation between interfering capacity and content of internal S antigen of different preparations (Paucker and Henle, 1958).

A true evaluation of the relative interfering capacity of incomplete virus is complicated by the fact that all native preparations contain some infectious virus as well (von Magnus, 1951b; Gard *et al.*, 1952). Recourse can be taken to systems in which even infectious influenza virus is capable of only limited reproduction. This approach has revealed that incomplete virus is not as potent an interfering agent as infectious virus. Thus, incomplete influenza virus derived either from mouse brain or from undiluted passage series in eggs gives no or negligible interference in mice against intracerebral doses of WEE virus, while equivalent amounts (in terms of HA titer) of active standard virus are highly effective (Schlesinger, 1951). More recently, Manire (1957) found a similar discrepancy in the capacity of standard and incomplete influenza virus to inhibit toxicity of intravenously administered homologous or heterologous influenza virus.

The findings of Henle and associates (Finter *et al.*, 1955; Paucker and Henle, 1955a,b; Liu *et al.*, 1956; Paucker and Henle, 1958) confirm substantial functional similarities between spontaneously arising incomplete and artificially inactivated influenza virus. Axiomatically, different preparations of either type are bound to be heterogeneous and to have variable populations. It is doubtful whether interference experiments or the crude technique of undiluted passage series would reveal subtle differences between incomplete and inactivated particles. The genetic approach suggests the existence of such differences. Burnet *et al.* (1954), in confirming the observation of Henle and Liu (1951) that partially inactivated virus is capable of multiplicity reactivation, at the same time find that incomplete virus can contribute genetic markers to progeny emerging from de-embryonated eggs doubly infected with incomplete and active virus, and in this respect resembles heat-inactivated virus (Burnet and Lind, 1954a). Gotlieb and Hirst (1956) find, in contrast, that partially UV-inactivated virus can be reactivated by double infection with active virus, while incomplete undiluted passage or mouse brain virus cannot. Perhaps these conflicting findings are again related to the variations of S antigen (Lief and Henle, 1956) and RNA (Ada and Perry, 1956) contained in different preparations of incomplete virus. It is clear that much more work has to be done along such lines before the interfering capacity of an incomplete particle can be clearly defined relative to that of an inactivated particle. Perhaps greater emphasis on systems employing heterotypic

influenza strains will aid in this. Up to now, most of the pertinent studies have been done with homologous systems, and here interference is so interwoven with the genetic potential of incomplete virus production that conclusions are difficult to draw.

C. Demonstration of Interference in Tissue Culture

1. *Interference in Surviving Tissue Fragments*

Prior to the recognition of cytopathogenic changes in growing tissue cultures as an index of viral activity, minced tissues in suitable media were employed occasionally for studies on interference (Andrewes, 1942; Huang, 1943; Lennette and Koprowski, 1946; Schlesinger, 1951). The viral combinations involved in these investigations are included in Table I. A true forerunner of the more sophisticated work with growing cultures is the work of Huang (1943), who recognized the pH change associated with actively metabolizing tissue fragments and used its inhibition as an indicator of cell destruction by viruses. Utilizing this tool, he observed that nondestructive agents, such as SLE or EMC viruses, effectively blocked metabolic inhibition due to EE virus.

2. *Interference in Growing Cell Cultures*

Actively proliferating cell cultures have provided an ideal tool for the detailed study of heterologous, homologous, or autointerference. At the qualitative level, i.e., in terms of inhibition of cytopathogenic effects, interference has been demonstrated for the following viral combinations: homotypic or heterotypic polioviruses (Le Bouvier, 1954; Sabin, 1954; Sabin *et al.*, 1954; Ledinko and Melnick, 1954; Drake, 1958), NDV and poliovirus (Chanock, 1955), Japanese B encephalitis and poliovirus (Mason and Woodie, 1955), influenza and EE (Taylor, 1953), NDV or influenza and WEE (Levine, 1958), UV-NDV and active NDV (Baluda, 1957), homologous and heterologous vesicular stomatitis virus (VSV) (Cooper, 1958), and a number of others for which documentation at the time of this writing is inadequate (Okuno *et al.*, 1956). Autointerference has found expression either in the form of depressed cytopathogenicity of large as compared with small doses of WEE virus in L cells (Chambers, 1957) or of inhibition of plaque formation by concentrated inocula of influenza (Granoff, 1955a) or VSV (Cooper, 1958).

Perhaps of the greatest interest and promise are observations on resistance of long-term virus-carrying cells to superinfection with homologous or heterologous viruses. Thus, Cieciura *et al.* (1957) found that less than 0.1 % of NDV-infected HeLa cells in monolayers survived. Such cells were grown to mass culture from which single cells were isolated. These were then permitted to form pure clones, and the process was repeated for over two years through

hundreds of generations. Throughout these manipulations, the cells were resistant to the killing effect of active NDV. Seeding such resistant cells on X-irradiated feeder layers caused massive destruction of both carrier cells and feeder cells. According to G. Henle *et al.* (1958), MCN or Lung-To cells persistently infected with NDV or mumps virus can be obtained and grown easily and at will. Such cell cultures yield about one infectious unit per 100 cells, suggesting either that only an occasional cell is producing virus or that all cells produce virus at an exceedingly slow rate. In favour of the latter hypothesis are two facts: (a) that nearly all cells are relatively resistant to reinfection with VSV, (b) that the "carrier" cultures exhibit a higher rate of aerobic glycolysis than uninfected cells (Green *et al.*, 1958). When VSV-challenged "carrier" cells are incubated for prolonged periods of time with proper refeeding at intervals, the challenge virus eventually (after 12 to 18 days) "breaks through" and multiplies to titers nearly equivalent to that obtained in control cultures (Bergs *et al.*, 1958). This observation indicates that VSV is not initially prevented from combining with the cells. Along similar lines, the resistance of L cells persistently infected with WEE virus to homologous superinfection (Chambers, 1957) and Ackermann's findings on HeLa cells with poliovirus (Ackermann and Kurtz, 1955) should be mentioned.

This approach, especially combined with the application of plaque assay methods for quantitative analysis, is in its infancy and undoubtedly will be pursued vigorously in the immediate future. It is an exciting prospect, for from studies of this sort we should learn much about the dynamics and the mechanism of interference. The stage is set for the analysis of events taking place in the single "interfered" cell. At the same time, interference here is a most valuable tool for the recognition of latent or persistent association of animal viruses with their host cells. How the knowledge of persistent infection of tissue culture may bear on our ideas concerning broader implications of interference is discussed above (see Section III, A, 1, *b*).

IV. Dynamics of Interference—Summary

Criteria for a rational approach to a comprehensive definition of interference are presented in the introductory section. Of all the experimental systems reviewed, only those utilizing myxoviruses as interfering agents have been analyzed sufficiently to offer some clues to their validity.

A. Qualitative Criteria—Specificity of Induction

The basic assumption is that interference is the expression of a specific change brought about by the association of susceptible cells with a virus. What is the experimental support for this assumption? Only the viral

particle itself, whether active or inactivated, has thus far been found capable of triggering the mechanisms resulting in interference. The chief evidence, other than quantitative correlations (see below), is as follows (Henle and Henle, 1945b): (a) neutralization of interfering capacity by homologous antiviral immune sera; (b) sedimentation of the interfering component along with viral particles; (c) adsorption on and elution from fowl red cells along with viral particles. Similar lines of evidence have been supplied by others for influenza (Ziegler *et al.*, 1944) or NDV (Baluda, 1957). Data on the effects of inactivating agents have significant, if more indirect, bearing on the subject. Henle and Henle (1947) first demonstrated that interfering capacity of influenza virus was less sensitive to UV-irradiation than infectivity or toxicity, but more so than HA or CF activities. The relative effect of heat is similar (Isaacs and Edney, 1950a). Baluda (1957) found that UV-irradiated NDV was equally effective as interfering agent regardless of whether it had received an average of 10, 50, or 100 units per particle. Powell and Pollard (1956) calculated from data on the effect of ionizing radiation that only a minute fraction of the influenza particle was responsible for interference. The nature of this fraction remains unidentified, and Paucker and Henle (1958) have been unable to induce the phenomenon with viral HA or S antigen obtained by disintegration of virus particles with ether. The suggestion that incomplete and standard particles interfere equally well with active influenza viruses is in line with comparable radiation sensitivity found for the two kinds of virus (Powell and Pollard, 1956).

The only hint that materials other than viral particles themselves may play a role in interference comes from the work by Isaacs *et al.* (1957) and Lindenmann *et al.* (1957) on "interferon," a macromolecular substance produced in and liberated from allantoic cells exposed to heat- or UV-inactivated influenza virus.[1] Although the rate of interferon production corresponds closely to that at which new viral antigens appear after infection with active virus, this material has no recognizable viral properties; it is not neutralized by antiviral immune serum; it is heat-labile (60°C. for 1 hour); it is not inactivated by RNAase, partially by trypsin. Isaacs (personal communication) believes it to be protein in nature, with no evidence for nucleic acid; it is not sedimentable by centrifugation at 100,000*g* for 30 minutes, but is retained by gradocol membranes APD 0.6μ. This suggests an asymmetrical, perhaps filamentous, shape. Interferon, added to normal CAM fragments prior to challenge with homologous or heterologous viruses (including vaccinia virus (Depoux and Isaacs, 1954a,b)), inhibits multiplication of the latter.

[1] An earlier observation by Lennette and Koprowski (1946) should be mentioned: The supernatant fluid or tissue extract prepared from 24-hour tissue cultures infected with 17DD yellow fever virus contained non-viral material (passing ultrafilter 12 mμ APD), which inhibited multiplication of West Nile virus. Unfortunately, this observation was not followed up.

It is too early to define the nature of interferon. It is not a self-perpetuating agent, but arises only in tissue exposed to inactivated virus. In this sense, its demonstration does not negate the basic premise that interference is induced by virus. But it poses a problem in the interpretation of results obtained under conditions where the interfering virus is added to the cell population at a multiplicity of less than 1 per cell.

B. Quantitative Criteria

1. *Number of Interfering Particles per Cell*

Fazekas de St. Groth and Edney (1952) calculated that a single particle of inactivated influenza virus per cell could induce heterologous interference. The same conclusion was reached by Baluda (1957) for homologous interference by UV-NDV. In this system, the calculated values were put to experimental test by enumerating viral particles before irradiation by plaque assay. On the other hand, for heterologous interference with two distinct strains of vesicular stomatitis virus, "many more than one inactivated particle" were required (Cooper, 1958).

2. *Speed and Duration of Interference*

According to Baluda (1957), the rapidity with which interference by UV-NDV with active NDV is established in tissue culture cells depends on the number of inactive particles adsorbed per cell. At very high multiplicity (140 particles/cell), the time required to induce exclusion may be as low as 0.1 minute. A single particle may establish interference after 6 minutes. With inactivated influenza virus, interference in individual cells may not be established for a much longer period of time, probably about 16 to 24 hours (Fazekas de St. Groth *et al.*, 1952; Henle and Paucker, 1958). Cooper (1958), using monolayers and plaque assays, found exclusion between heterotypic VSV strains operating after 12 minutes. The situation appears to be fundamentally different in the case of interference by NDV (or PR8) with WEE virus (Levine, 1958). Here, there is a mutual depressing effect of the two viruses involved. That is to say that the ability of NDV-infected cells to produce WEE virus decreases exponentially with time after NDV infection; at the same time, WEE virus, added to cells up to 5 to 6 hours after infection with NDV, can reduce the amount of NDV produced. Cells infected with PR8 virus lose their ability to produce WEE virus only after 4 to 5 hours.

The duration of interference depends, of course, on the experimental system used and, specifically, on the nature of the association between interfering virus and cell. In the most critically studied system, that of Baluda (1957), exclusion of NDV by UV-NDV-infected cells disappeared after 26 to 60 hours. For influenza viruses in eggs, it was found earlier that

interference was effective 6 days after inoculation of inactivated virus (Henle, 1950). Since basically nothing is known about the nature of the association of these inactivated agents with their host cells, one cannot say what factor is responsible for the ultimate cessation of interference. On the other hand, the persistence of exclusion of superinfecting viruses from long-term "carrier" cells (see Section III, C, 2) suggests that interference is effective as long as the interfering agent remains actively associated with the cell. This statement implies nothing in regard to any possible analogy of such systems to lysogeny which has not been proved in a single case.

3. *Efficiency—"Overcoming" of Interference*

Baluda (1957) finds that "there exists for each interfered cell a small probability that it can be superinfected by any given active virus particle. From the results obtained, either the maximum value of this probability is very small for all the cells, i.e., less than 4 %, or it has a value of 1 for a small fraction of the cells only." This statement implies, of course, that the chances of "overcoming" interference increase with the multiplicity of superinfecting viral particles.

The corresponding situation for influenza virus has not been as clearly defined. As indicated, establishment of solid interference requires up to 16–24 hours. Whether the gradually decreasing yield of progeny of superinfecting virus up to that time is due to "bypassing" (Fazekas de St. Groth *et al.*, 1952) or to reduced yield from all cells is uncertain. Evidence in favor of the former view may be invalidated by use of maximally effective interfering virus (Henle and Paucker, 1958). In contrast to the NDV system, increased multiplicity of superinfecting virus does not significantly increase the final yield (Henle and Henle, 1944b; Fazekas de St. Groth *et al.*, 1952). A complicating factor, already mentioned, is the theoretical possibility that some apparent "overcoming" in homologous influenza systems may in fact be due to reactivation and recombination between interfering and superinfecting virus. Isaacs and Edney (1951a) observed strain-specific differences in the case with which different variants of type A strains could "overcome" interference induced by a standard dose of heated LEE. They thought at the time that this indicated that complete interference could not necessarily be accounted for by the assumption that 1 particle of heated LEE per cell assured interference. This is perhaps one situation in which further studies on interferon (Isaacs *et al.*, 1957) will clear up apparent quantitative discrepancies.

V. Mechanisms of Interference

A uniform approach to the theory of interference is impossible. Instinct tells us that the mechanism whereby influenza virus interferes with homologous

or heterologous myxoviruses must differ from that by which the same virus inhibits multiplication, say, of equine encephalitis virus. What are the differences? What is the residual biological activity of an "inactivated" virus particle that can have such a profound effect on the cell's response to superinfecting virus? What possible justification—other than our ignorance about mechanisms—can there be for classifying together under a single term two phenomena as manifestly different as the resistance to EE virus of mice infected with Theiler virus and the refractoriness to superinfection of single cells in a clone persistently infected with NDV? Are some of the phenomena which we call interference simply due to the destruction of susceptible cells by one of two pathogenic viruses, cf. TO-WEE, while at the other extreme we are dealing with relationships as intimate as lysogeny in phage-infected bacteria?

Even in the case of bacterial viruses, basic mechanisms involved in interference remain unsolved. It has been possible, however, to differentiate some expressions of mixed or multiple infection by descriptive terms with specific connotations. *Mutual exclusion* is the resistance of infected cells to superinfection with a related or unrelated virus; the *depressor effect* is the lowering of the yield of the first infection by the superinfecting and excluded phage; *immunity of lysogenic bacteria* to superinfection follows laws of specificity differing from those governing mutual exclusion between virulent phages. Techniques are available by which these phenomena can be differentiated from reactivation or genetic recombination or phenotypic mixing. Nevertheless, making these distinctions will be greatly complicated if, for example, it should ever prove that one viral mutant has a distinct selective advantage over another one; or, if mixed infection with N related phages, each having distinct genetic markers, should reveal that fewer than N particles can participate in the production of progeny in a single infected cell. Despite such theoretical reservations, it is clear even now that in multiple or mixed infection of bacteria a variety of yield-limiting mechanisms may operate whose recognition has given rise to a usable descriptive terminology.

The fact that, in the case of animal viruses, we lump together so many diverse phenomena under the single heading of "interference" merely attests to the lack of basic information necessary for a more discriminating terminology. In a purely descriptive sense, one is tempted to see analogies to mutual exclusion, depressor effects, or lysogeny, and to add to these a number of other hypotheses. At this time, however, there is little solid experimental support for any definitive differentiation. What little evidence there is, has been derived either directly or indirectly from studies on adsorption and penetration of interfering or suppressed myxoviruses.

A. Nature of Association of Interfering Virus and Cell

Inactivated, interfering influenza virus is adsorbed and "disappears" in the allantoic membrane much as active virus does (Henle and Henle, 1944b;

Henle *et al.*, 1947a). It is of interest that Tyrrell and Tamm (1955) reported that interference by heat-inactivated influenza virus could be inhibited by 2, 5-dimethylbenzimidazole, a compound thought to have an effect on the nucleic acid metabolism. Interference by mustard- or heat-inactivated virus was slightly inhibited by cortisone (Fong and Louie, 1953; Kilbourne, 1957). Evidence obtained from radiation data suggests that the interfering moiety of influenza virus resides in a minute fraction of the viral particle, perhaps associated with the RN-protein (Powell and Pollard, 1956; Powell and Setlow, 1956). There is, however, no direct experimental basis for implicating viral genetic material in the establishment of interference, except insofar as the material reviewed above points to close functional relationship between interfering capacity, "completeness" of virus, and ability to contribute genetic markers to recombinant progeny. Recent experiments, though confirming the inhibitory effect of RNAase on multiplication of influenza viruses (LeClerc, 1956), have failed to reveal inhibition by RNAase of interference by UV-inactivated virus with active heterologous virus (Schlesinger and Kuske, 1958).

In contrast to the evidence pointing to intracellular penetration of interfering influenza viruses, results of Baluda (1957) suggest that establishment of interference by UV-NDV with active NDV in tissue culture may follow upon superficial attachment of the inactive particles. In this system, treatment of monolayers with anti-NDV antibody as late as 90 minutes after adsorption of UV-NDV can prevent exclusion in about 50 % of the cells. "Carrier" clones of pure cell lines persistently infected with NDV are refractory to superinfection with NDV (Cieciura *et al.*, 1957) or VSV (G. Henle *et al.*, 1958). Although the finding of an increased rate of aerobic glycolysis in such carrier cells (Green *et al.*, 1958) points to a fundamental, virus-induced alteration in their function, even they can be "cured" of infection by treatment with anti-NDV immune serum (G. Henle *et al.*, 1958). Thus, the ability to remove NDV, and with it resistance to reinfection, from tissue culture cells suggests that this system is not analogous to lysogeny in bacteria. But whether the difference between influenza virus and NDV is of fundamental nature or perhaps reflects the different physiological state of tissue culture as compared with allantoic cells remains to be seen.

B. Fate of the Superinfecting Virus

Under conditions of interference in the allantoic membrane, adsorption and "disappearance" of homologous or heterologous active influenza virus is not prevented (Henle *et al.*, 1947a; Isaacs and Edney, 1950a,c). Moreover, when effective interference has been established, the amount of mucoprotein HA inhibitor extractable from allantoic membrane is undiminished (Liu and

Henle, 1951a; Edney and Isaacs, 1950). On the basis of this evidence, Henle (1950) and Isaacs and Edney (1950b,c) postulated that interference in the influenza-influenza system is probably localized at an intracellular site beyond the stage of adsorption. This interpretation is certainly in line with the established close relationship between interference and genetic recombination or reactivation which indicates that pre-emption of a cell by inactivated viral particles does not exclude invasion by active particles. If we could identify the fundamental difference between the conditions enabling an inactivated particle to contribute genetic markers to recombinant progeny and those leading to interference, we would be a good deal closer to an understanding of mechanisms.

Another system for which experimental evidence strongly points to an intracellular mechanism is that concerned with interference by active myxoviruses with EE virus. Here it was shown by Schlesinger (1951) that the cellular receptors for influenza virus could be effectively destroyed by treatment with RDE without affecting susceptibility of tissue fragments to EE virus. More recently, Levine (1958) confirmed this observation by showing that monolayers of chick embryo fibroblasts infected with NDV attached the same number of plaque-forming units of WEE virus as uninfected monolayers. Levine's quantitative data, already described, suggest that interference in this system may involve direct competition for cellular constituents (or for limited sites?) required for replication of both viruses. The idea of direct mutual competition is in line with the early observation by Vilches and Hirst (1947) that suppression of a slowly multiplying strain of WEE virus was much more effective than that of a rapid mutant. Furthermore, large doses of UV-irradiated (Henle and Henle, 1945a; Vilches and Hirst, 1947) or of "incomplete" influenza virus (Schlesinger, 1951) do not effectively interfere with WEE virus. All these considerations point indeed to mutual intracellular inhibition by two actively multiplying viruses.

In contrast to the inferred intracellular localization of interference by influenza viruses, Baluda's (1957) evidence concerning the fate of superinfecting NDV or UV-NDV-infected cells again suggests that he is dealing with a surface phenomenon. He has shown that superinfecting particles disappear from "interfered" cells more rapidly than from normal ones. That fraction which remains attached can be inactivated by antibody at a time when virus in association with normal cells would no longer be neutralizable because it has penetrated. Baluda therefore suggests that active NDV, after adsorption on "interfered" cells, is either inactivated at the cellular surface or prevented from penetrating into the cell. Application of isotope techniques may help decide between these alternatives, much as it has in the case of mutual exclusion between phages (French *et al.*, 1951).

C. Perspectives

It seems presumptuous to speculate on mechanisms of interference at a time when the field is conceptually far ahead of its methodology. It is reassuring that new methods are rapidly becoming available with which to test various hypotheses. Critical work on interference, as on other phases of the infectious process, depends on quantitative assay methods, study of single cell yields, genetic analysis of viral progenies, and biochemical characterization of the viral life cycle. The increasing weight of evidence for the essentiality of viral RNA as the basic multiplying unit of various animal viruses (Colter *et al.*, 1957; Wecker and Schäfer, 1957) may direct future efforts toward analogies to mechanisms controlling biosynthesis of constitutive or induced enzymes. As seems to be the case in interference, so here these mechanisms are concerned with permeability problems (Cohen and Monod, 1957) as well as direct competitive inhibition (Cohn, 1957). At another elementary level, further knowledge on interference between animal as well as bacterial viruses will undoubtedly be stimulated by the findings on interference between DNA preparations in bacterial transformations (Hotchkiss, 1954; Alexander *et al.*, 1954) which, according to a recent report by Schaeffer (1957), is due to inability of the DNA to penetrate into "interfered" bacterial cells. Ultimately, the evolution of a unified concept of interference will come when the single mammalian cell, uninfected or virus-infected, has become a well-defined, functional, genetic, and biochemical entity.

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Chapter VIII

Inhibition of Multiplication

FRANK L. HORSFALL, JR.

The Rockefeller Institute for Medical Research New York, New York

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I. INTRODUCTION

Contrary to common opinion, it is not difficult to inhibit the multiplication of some animal viruses. The dynamics of the multiplication process and the yield of new virus particles are affected by a wide variety of alterations in the environment and metabolism of the infected host cell. They also are affected by a diverse array of chemical substances that are more or less active as inhibitors of multiplication.

The genotype and age of the host, its nutritive state, the supply of vitamins and other food factors, environmental conditions, infection with bacteria or

with viruses, and numerous other variables may alter the process of virus multiplication. As a general rule, the more abnormal the environmental or metabolic conditions, the more marked is the inhibitory effect on multiplication.

A large number of biological materials and chemical compounds affect the multiplication of animal viruses. The great majority of these substances tend, in widely varying degree, to inhibit the process. A few substances are known to have a different effect and lead to augmentation of multiplication. None of the inhibitory substances presently known has been demonstrated to be useful in the management of naturally occurring virus infections of animals or man.

The effects of various biological materials and chemical compounds on virus multiplication have been the subject of recent reviews by Matthews and Smith (1955), Horsfall (1955a,b), Hurst and Hull (1956), Tamm (1956a, 1958), Horsfall and Tamm (1957.) No attempt will be made to present a complete evaluation of the properties and capabilities of the large number of inhibitory materials that have been reported. The present discussion is concerned largely with the possibility that principles bearing on inhibition can be discerned and that conceptual schemes erected on such bases may be useful in furthering investigations on the mechanism of virus multiplication.

The multiplication of animal viruses has been approached in different ways by different workers. Some have not made a sharp distincton between multiplication and its incidental sequelae—lesions and disease. Others have been concerned exclusively with the pathological alterations that sometimes are the end results of multiplication. Relatively few have focused attention closely on the processes which lead to the production of new virus particles in the infected cell. During recent years, ideas that have evolved from the detailed study of the reproduction of bacterial viruses have strongly influenced experimentation and concepts in the animal virus field. Much modern work on the multiplication of animal viruses is similar in plan and objectives to that with bacterial viruses and has led progressively to the view that the mechanisms involved have much in common.

Studies with inhibitory materials have, in many cases, suffered from the failure to make a clear distinction between virus multiplication per se and the effects of the process or its products upon host cells and tissues. In many reports little or no attention has been given to the mechanism of inhibition. In some it has been assumed that, because the incidence of lesions or death of the animal was reduced when a compound was used, multiplication of the virus must also have been inhibited. Such an assumption is unwarranted and may be in error. To determine whether a substance inhibits multiplication it is essential to measure the rate or the extent of multiplication that occurs in its presence and compare this with the results of identical measurements

made in the absence of the substance. It is surprising how infrequently this direct approach has been employed.

There are, it appears, a number of discrete steps in the complex process of virus reproduction. Present evidence indicates that some inhibitory substances act to alter one step but may not affect others. In most instances, however, there is little information that bears directly on the mechanism of inhibition and it is difficult to discover whether virus attachment, penetration, intracellular biosynthesis, assembly and maturation, or release were affected. It will be apparent that these terms, which identify certain of the well-recognized steps in the multiplication process, come from the current scheme concerning bacterial viruses. A diminished yield of virus may indicate that inhibition has been produced, but it leaves unanswered the more important question: What process was inhibited? Much more rewarding information is secured when studies are designed to provide information about the step or steps in the multiplication process that are affected.

When study of the inhibition of virus multiplication is correlated as closely as possible with studies of the multiplication process per se, new information on either problem may contribute to advances regarding the other. From the broad viewpoint these two aspects of the problem are so closely related as to be almost inseparable and probably are most effectively approached simultaneously.

Throughout this chapter, a sharp distinction is made between prevention of infection, on the one hand, and inhibition of intracellular multiplication, on the other. This is considered to be important because both may lead to an identical operational result, i.e., a diminished yield of new virus particles, even though the mechanisms involved may be wholly dissimilar. Substances which are highly effective in preventing infection, e.g., specific antibody, may have little or no effect upon intracellular multiplication. Similarly, substances which are very potent inhibitors of intracellular multiplication, e.g., certain chloro-glycosyl derivatives of benzimidazole (Tamm, 1956a) may be entirely incapable of preventing infection of a susceptible host cell.

By design, an effort is made also to distinguish clearly between the fundamental biological unit, i.e., the single virus-infected cell, and the experimental material which is, in the great majority of instances, either a tissue culture system, a separated tissue, or an intact animal infected by a virus. As will be evident, this distinction is possible less commonly than is desirable in experiments with inhibitory materials. When inferences can be drawn at the level of the virus-infected cell, many of the uncertainties which stem from assumptions made in studies with the intact animal host are removed. Seemingly crucial variables, such as the size of the inoculum, the time between inoculation and administration of the inhibitory substance, the presence or absence of gross lesions, and others, have little relevance to events occurring

in individual virus-infected cells. Because animal virus multiplication, like that of bacterial viruses, is a process that is wholly completed within the individual, infected host cell, inhibition of multiplication needs to be examined at the cell level.

II. Prevention of Virus Infection

Infection can be considered to have begun when the infecting virus particle or a part of it has penetrated into a host cell that can support multiplication. To prevent infection it is necessary to alter the extracellular environment, the virus particle, or the host cell so that one or more of the steps leading to penetration do not occur. If infection of all susceptible cells is prevented, no multiplication occurs and no new virus particles are produced. If infection is not completely prevented, a variable fraction of the susceptible cells supports multiplication, and new virus particles appear in a yield that is smaller than that expected in relation to the total population of susceptible cells. The numerical result is identical to that secured when intracellular multiplication is partially inhibited but not entirely blocked.

On theoretical grounds it should be possible to prevent infection by: (1) altering the extracellular environment so that attachment of virus particle to susceptible cell will not occur; (2) inactivating the infective property of the extracellular virus particle; (3) altering the host cell so that either attachment of the virus particle or penetration of its functional part does not occur.

A. Alteration of Extracellular Environment

To prevent infection by alteration of the extracellular environment is relatively difficult in the intact animal, but is readily accomplished with certain animal viruses in tissue culture. As with bacterial viruses, it appears probable that the initial bond formed in attachment of animal viruses to cells is electrostatic in nature (Tolmach, 1957). It would be expected that such a bond would exhibit salt dependence and would show reversibility. A sufficient decrease in the electrolyte concentration of the extracellular environment has been shown to prevent attachment of influenza virus or pneumonia virus of mice to erythrocytes (Davenport and Horsfall, 1948). Similarly, pneumonia virus of mice (Davenport and Horsfall, 1950) and Newcastle disease virus (Levine and Sagik, 1956) are prevented from attaching to host cells if the salt concentration is adequately reduced. The nature of the ionic environment, particularly the type and concentration of cations, also is important for attachment (Burnet, 1952). Although binding to host cells is independent of pH over a wide range, with Newcastle disease virus it is inhibited at pH 4 or 10.5 (Levine and Sagik, 1956). It needs to be emphasized that in all instances

it is necessary to produce gross and very unphysiological alterations in the electrolyte environment to prevent attachment.

In theory, reversal of attachment should also prevent infection. The period during which reversibility is demonstrable may be brief and the phenomenon difficult to demonstrate with animal viruses, but there are indications that it exists. Cation exchange resins (Puck and Sagik, 1953) can reverse the attachment of influenza virus to erythrocytes. A sufficient increase in temperature reverses the attachment to cat erythrocytes without causing destruction of cell receptors (Tamm, 1954a). Similarly, reduction in the electrolyte concentration reverses the attachment of pneumonia virus of mice to host tissue particles or erythrocytes without affecting the combining power of either (Davenport and Horsfall, 1948, 1950).

B. Inactivation of Extracellular Virus

Inactivation of the infective property of extracellular virus particles is by far the simplest and the most generally used means for preventing infection of susceptible host cells. Specific neutralizing antibody is the antiviral substance commonly used and is much more effective than any other. In the great majority of instances, neutralizing antibody converts infective virus particles into noninfective particles at a rapid rate and with an efficiency that approaches but does not quite reach 100 % (Dulbecco *et al.*, 1956; Rubin, 1957). It appears that a small fraction of particles resists neutralization, but no satisfactory explanation has been provided for this so-called persistent fraction, which was recognized much earlier in studies with bacterial viruses (Andrewes and Elford, 1933; Burnet *et al.*, 1937). Recent studies (Dulbecco *et al.*, 1956; Rubin, 1957) have been interpreted as indicating that the persistent unneutralized particles are in no other way demonstrably different from the particles that are neutralized; they appear to have the same antigenic make-up, are not genotypically different, and on multiplication in susceptible cells yield new particles that react with neutralizing antibody to the same extent as did the initial population of virus particles.

The mechanism by which neutralizing antibody inactivates the infective property of viruses is not known. There are indications that neutralization is a direct result of combination between virus particle and antibody—that the binding of one antibody molecule by a virus particle may cause inactivation (Dulbecco *et al.*, 1956). There is also evidence for the view that antibody inactivates virus particles by preventing penetration of susceptible cells by the agent (Rubin and Franklin, 1957; Rubin, 1957). The kinetics of inactivation in the presence of antibody excess is of first order during the initial phase of the reaction; the rate is directly dependent on the concentration of antibody.

Recent work with bacterial viruses (Lanni and Lanni, 1953) and with plant viruses (Schramm and Gierer, 1957) indicated that neutralizing antibody

reacts with the protein components of the virus particle, not with the separated nucleic acid which carries the functional capacity to initiate reproduction in an appropriate host cell. There are indications that point in the same direction with animal viruses; the separated infective nucleic acid fraction of Mengo virus (Colter *et al.*, 1957) is not inactivated by neutralizing antibody, which does inactivate the infectivity of the intact virus particles. These results raise the possibility that virus particles neutralized by specific antibody may retain the intrinsic capacity to initiate multiplication but are prevented from manifesting it through inability either to bind with host cells or to penetrate the cell membrane. In support of this idea, Isaacs (1948) has demonstrated that anti-influenza virus serum prevents attachment of this virus to erythrocytes. The well-established fact that neutralizing antibody does not alter the kinetics of the intracellular multiplication process after the virus has penetrated the host cell can be taken as confirmatory evidence. The work of Watson and Coons (1954) leaves little doubt that antibody can penetrate host cells, but it is well established (Ginsberg and Horsfall, 1951b) that it does not produce any effect upon the intracellular virus particle or its functional part comparable to that it produces on the intact extracellular virus particle.

The question whether neutralizing antibody and virus particle can dissociate with apparent reactivation of the infective property seems not to be decisively resolved. Certain studies (Tyrrell and Horsfall, 1953) indicate that reactivation of the infectivity of a neutralized mixture occurs on dilution. Others (Dulbecco *et al.*, 1956) have secured contrary results. It must be emphasized that infectivity is a positive operational concept that has meaning only with respect to particular host cells under specified environmental conditions. Noninfectivity is, in contrast, a negative concept which has meaning only in relation to the conditions specified. The difficulties of a decision regarding noninfectivity produced by neutralizing antibody have been underscored by Tyrrell and Horsfall (1953).

Many materials have been tested with a view to finding means other than antibody to inactivate extracellular virus particles *in vivo*. The extraordinary instability of certain animal viruses *in vitro*; some, i.e., influenza and mumps, have an infective half-life that is no longer than an hour or two 35°C. (Horsfall, 1955c), suggests that their inactivation should be readily achieved. Numerous substances are effective *in vitro*, and appear to inactivate virus particles directly, but nearly all are so damaging to host cells as to prevent their use *in vivo* (Horsfall and Tamm, 1957). An exception is provided by synthetic polylysine peptides (Green and Stahmann, 1954), which appear to combine directly with a number of animal viruses, i.e., influenza, mumps, Newcastle disease, and infectious bronchitis, as well as with tobacco mosaic and certain bacterial viruses, and inhibit their infectivity while they are

extracellular. Similarly, β-ethoxy-α-ketobutyraldehyde hydrate (Kethoxal) appears to have a direct virucidal action on a variety of animal viruses, i.e., influenza, Newcastle disease, mumps, and vaccinia (McLimans *et al.*, 1957).

C. Alteration of Host Cells

There is some evidence that infection can be prevented or retarded by appropriate alteration of the host cell. Among animal viruses most of the studies directed toward this possibility have been done with influenza. This virus has been used because of the information which has been accumulated regarding the so-called virus receptor of the host cell (Burnet, 1955; Tolmach, 1957).

Treatment with receptor-destroying enzyme derived from *Vibrio cholerae* (Burnet *et al.*, 1946), has been shown to alter susceptible cells in the allantoic sac of the chick embryo (Stone, 1948a); in the mouse lung (Stone, 1948b); and in the mouse brain (Cairns, 1951). The host cell alteration produced by the enzyme leads to a significant degree of prevention of infection when appropriate strains and inocula of influenza virus are employed (Burnet, 1955). It appears that the enzyme does alter the receptors of the host cell, for it has been demonstrated that after treatment *in vivo*, the excised mouse lung shows a reduced capacity to adsorb the virus (Fazekas de St. Groth, 1948a). The receptors apparently are not permanently destroyed, regardless of the extent of treatment with the enzyme, and are capable of regeneration some hours after its removal (Fazekas de St. Groth, 1948b).

Certain amino-sulfonic acids (Ackermann, 1952), particularly α-amino-*p*-methoxyphenylmethanesulfonic acid (Ackermann and Maassab, 1954a,b), may also alter host cells and prevent infection with influenza virus. These compounds do not inactivate the virus and do not affect cell respiration in tissue culture. The compound identified above appears to affect an early stage in the process of infection and possibly prevents penetration. It has apparently no effect on the intracellular multiplication process per se, although it appears to affect the release of mature virus particles from the infected cell.

Numerous other substances have been considered as having some effect on host cells that modifies their capacity to be infected by animal viruses. Comments on these substances are found in the recent reviews by Matthews and Smith (1955) and Hurst and Hull (1956). In the large majority of instances there is not sufficient information to make possible a reasonable decision on the mode or site of action.

III. Inhibition of Intracellular Multiplication

The preliminary steps in the initiation of infection by viruses have as their goal the development of a unique biological entity, the virus-infected cell. Attachment of virus particles to host cells and penetration of the virus or its

functional part into the host cell do not in themselves result in infection. This develops only if the virus multiplies—a point of view that has been emphasized recently (Horsfall and Tamm, 1957; Horsfall, 1958). If the virus is to multiply or reproduce, the metabolism of the infected host cell must be altered and redirected so that certain of the products of cell biosyntheses are different from those of the noninfected cell.

The virus-infected cell is unique because it produces new virus particles; these contain components which are not found in and presumably are not produced by the noninfected cell. In the absence of contrary evidence, it can be assumed that the unusual and new orientation of some of the biosynthetic processes of the virus-infected cell is initiated by the virus particle or a part of it. Recent studies on the metabolism of bacteria infected with bacterial viruses (Putman, 1953; Cohen, 1955; Hershey, 1957) and on the mechanism by which infection is initiated with these viruses (Hershey, 1957) provide strong support for this view, if it be accepted that there are unifying principles in biology, and that one such principle concerns the mechanism of multiplication of viruses regardless of the nature of their hosts.

To inhibit intracellular multiplication of viruses it is necessary to affect biosynthetic processes in the virus-infected cell so that new virus particles are not produced in the expected yield. The objective is to prevent, retard, or diminish the effective use of those intracellular processes which are newly oriented in the direction of producing virus precursor materials. It should be emphasized that their potentiality to become so oriented is not discoverable until the cell has become infected. After the virus particle or a part of it has penetrated the cell, but not before, this dormant capability becomes evident, as is proved by the later appearance of new virus particles.

It is now commonly accepted by workers in the virus field that the biosynthesis of virus precursor materials in the infected cell occurs largely, if not exclusively, during the so-called latent period. This concept has received largest support and most extensive documentation from studies with bacterial viruses (Putnam, 1953; Cohen, 1955; Hershey, 1957), but there is evidence which points in the same direction with animal viruses (Horsfall and Tamm, 1957; Horsfall, 1958). If substances with inhibitory activity are effective because they diminish the production of virus precursor materials, such substances should show activity during the latent period. Relatively few substances have been studied with sufficient attention to the kinetic aspects of multiplication to make it feasible to decide at what step in the process they are active. Those which appear to affect processes occurring during the latent period are discussed below.

A. Inhibition during Latent Period

No more than seven substances have been conclusively demonstrated to inhibit multiplication during the latent period of animal viruses. These are:

(1) *Klebsiella pneumoniae*, type B, (Friedländer bacillus) capsular polysaccharide (Fr. B). which inhibits pneumonia virus of mice (PVM) reproduction (Ginsberg and Horsfall, 1951b); (2) 2, 5-dimethylbenzimidazole (MB), which inhibits influenza B virus multiplication (Tamm *et al.*, 1953a; Tamm and Tyrrell, 1954); (3) DL-methoxinine, which inhibits influenza A virus multiplication (Ackermann and Maassab, 1954b); (4) 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which inhibits influenza B virus multiplication (Tamm and Tyrrell, 1954) and poliovirus, type 2, multiplication (Tamm, and Nemes, 1957); (5) *p*-fluorophenylalanine (FPA), which inhibits poliovirus, type 3, multiplication (Ackermann *et al.*, 1954); (6) levo-γ-(*o*-chlorobenzyl)-δ-oxo-γ-phenyl caproic acid (caprochlorone), which inhibits influenza A virus multiplication (Liu *et al.*, 1957a); (7) 5, 6-dichloro-1-α-D-arabinopyranosylbenzimidazole (DAB) which inhibits poliovirus, type 2, multiplication (Tamm and Nemes, 1957).

To prove that a substance inhibits during the latent period, it is essential to use single-cycle multiplication experiments, comparable to the one-step growth experiment devised for bacteriophage (Ellis and Delbrück, 1939), and to add the inhibitory substance at various intervals after attachment and penetration of the virus. Perhaps because of the technical difficulties and large number of measurements that are required, such kinetic experiments have not been frequently carried out. The small number of substances known to cause inhibition during the latent period is probably attributable more to lack of adequate data than to special features of the substances that are known to be inhibitory during this interval.

1. *Klebsiella Pneumoniae, Type B, Capsular Polysaccharide (Fr. B)*

This substance inhibits the multiplication of pneumonia virus of mice (PVM) in the lung of the living mouse (Horsfall and McCarty, 1947a). In single-cycle multiplication experiments (Ginsberg and Horsfall, 1951b), 0.1 mg. per mouse, intranasally, markedly inhibited reproduction of the virus when given at 4, 8, or 10 hours, but not at 12 hours after inoculation. Because the latent period of PVM in the mouse lung is about 15 hours (Ginsberg and Horsfall, 1951a), it appears that the polysaccharide is effective as an inhibitor only during the first two-thirds of the interval. It seems clear on the basis of the time relationships that it does not act by preventing attachment of the virus or penetration of susceptible cells. Moreover, because it is ineffective during the last third of the latent period, it is apparent that it does not affect either the maturation of new infective virus particles or their eventual release from the virus-infected cell (Horsfall, 1952).

If the polysaccharide is given after the end of the latent period, e.g., at 18 hours, it has no effect on the yield of virus from the first cycle but effectively

inhibits multiplication in the next cycle, as would be expected from its activity during the latent period.

The substance does not inactivate the infectivity of the virus particle (Horsfall and McCarty, 1947b) and does not prevent adsorption of the virus by erythrocytes or lung tissue (Horsfall and McCarty, 1947a). Oxidation with periodic acid, sufficient to destroy specific serological activity, does not diminish the inhibitory activity of the polysaccharide (Horsfall and McCarty, 1947a) but does markedly diminish the toxic effects of the substance in the mouse lung (Ginsberg and Horsfall, 1951b). Although depolymerization has no effect on inhibitory activity, the aldobionic acid derived from the polysaccharide by acid hydrolysis is inactive (Horsfall and McCarty, 1947a).

All attempts to discover the biochemical basis for the inhibitory activity of *K. pneumoniae*, type B, capsular polysaccharide have been unsuccessful. The precise chemical structure of the substance has not been established. There are, however, good indications that its effect on host cells may be specific in character and dependent on a selective biochemical alteration. Although the polysaccharide is highly active as an inhibitor of multiplication of pneumonia virus of mice in the mouse lung (as little as 2 μg. given once causes 90 % inhibition), it has no effect on the multiplication of influenza viruses in the lung of the same host species (Horsfall and McCarty, 1947a; Ginsberg *et al.*, 1948). Moreover, although some other polysaccharides, including the capsular substances from *K. pneumoniae*, types A and C, and streptococcus MG, as well as blood group A substance and dextran, synthesized from sucrose *in vitro* by a cell-free enzyme, have similar inhibitory activity on the multiplication of PVM, numerous other polysaccharides do not (Horsfall, 1952). Among the latter is the capsular polysaccharide of pneumococcus, type 2, which, although related serologically to that of *K. pneumoniae*, type B, is wholly inactive as an inhibitor (Ginsberg *et al.*, 1948).

It is curious that the polysaccharide is effective as an inhibitor only when it is given intranasally in mice (Horsfall and McCarty, 1947a). This is also the only route of inoculation that leads to infection with PVM (Horsfall and Hahn, 1940). No adequate explanation for the decisive character of the route of administration has been offered, but it may be that the large size of the polysaccharide molecule prevents its diffusion into the lung in adequate concentration to yield inhibition when given by other routes. In support of this suggestion, it is known that the substance is poorly and irregularly transported from the yolk sac to the allantoic fluid in the chick embryo (Ginsberg *et al.*, 1948).

In addition to its inhibitory effect on the intracellular multiplication of PVM, *K. pneumoniae*, type B, capsular polysaccharide is a potent inhibitor of mumps virus multiplication in the allantoic sac of the chick embryo (Ginsberg *et al.*, 1948). Although there are some data indicating that the

substance may act during the latent period of mumps virus (Ginsberg and Horsfall, 1949), single-cycle multiplication experiments adequate to establish this point could not be carried out.

The polysaccharide not only inhibits the multiplication of PVM during the latent period, but it acts as a chemotherapeutic agent in the strictest sense and in the living mouse alters the course of infection with a small virus in favor of the host (Ginsberg and Horsfall, 1951b). Under appropriate experimental conditions, animals treated with a single injection of the substance, after lung lesions have appeared, recover from a virus infection which is uniformly fatal in control animals.

2. 2, 5-*Dimethylbenzimidazole* (*MB*)

This compound, designated MB, was used to initiate a systematic investigation of the inhibitory activity of derivatives of benzimidazoles relative to their chemical structure (Tamm *et al.*, 1952). It inhibits the multiplication of influenza A or B virus in the chorioallantoic membrane of the chick embryo *in vitro* (Tamm *et al.*, 1952).

In single-cycle multiplication experiments (Tamm *et al.*, 1953a; Tamm and Tyrrell, 1954) the compound at 0.38 mg. per milliliter inhibited reproduction of influenza B virus markedly when added at 1, 2, or 3 hours, and definite inhibition was obtained even when it was added at 4 or 5 hours after inoculation. It also inhibited the production of soluble complement-fixing antigen to a similar extent throughout the same interval. The later the compound was added during the latent period, the smaller was the inhibitory effect both on virus multiplication and production of soluble antigen (Tamm and Tyrrell, 1954).

The compound exerts an inhibitory effect throughout the latent period of influenza B virus and for at least an hour after the end of the latent period. The duration of processes inhibitable by the compound is therefore considerably longer than those inhibitable by DRB (Tamm and Tyrrell, 1954), which is described in a later section.

MB does not inactivate the infectivity of influenza virus *in vitro*, and does not decrease O_2 consumption by the chorioallantoic membrane. It does not cause an irreversible alteration in the host cells, for prolonged exposure to the compound does not diminish the capacity to support virus multiplication when the compound is removed (Tamm *et al.*, 1952). The biochemical basis for the inhibition produced is not yet known.

3. DL-*Methoxinine*

This compound inhibits the multiplication of influenza A virus in the chorioallantoic membrane of the chick embryo *in vitro* (Ackermann, 1951a). In single-cycle multiplication experiments (Ackermann and Maassab, 1954b),

1.3 mg. per milliliter completely suppressed virus increase when the compound was added at 1 or 2 hours, was considerably less effective when added at 3 or 4 hours, and had no effect on reproduction at 6 hours after inoculation. The latent period of influenza A virus in the chorioallantoic membrane *in vitro* is about 3 hours (Ackermann and Maassab, 1954a,b). The compound is most effective during the first two-thirds of the latent period but has some inhibitory effect during the last third of the latent period and a slight but definite activity even later in the cycle.

The inhibitory effect of the compound is blocked by L-methionine but not by D-methionine (Ackermann, 1951a). This result was taken to indicate that L-methionine is involved in the biosynthesis of influenza A virus and it was suggested that some function of methionine rather than its synthesis is inhibited by the analog (Ackermann, 1951a).

DL-Methoxinine does not inactivate the infectivity of the virus *in vitro*, does not prevent attachment to or penetration of host cells, and does not affect the endogenous respiration of the chorioallantoic membrane (Ackermann, 1951a). Moreover, the compound does not irreversibly impair the capacity of the tissue to support virus multiplication; on removal and addition of methionine, multiplication proceeds as in control membranes (Ackermann, 1951a). The results obtained with DL-methoxinine support the view that some phase-specific reactions exist and that one of these involves methionine. The function of this reaction appears to be completed before the appearance of the infectious property of the virus (Ackermann and Maassab, 1954b).

Methoxinine also inhibits the multiplication of vaccinia virus in chick embryo tissue *in vitro* (Thompson, 1947). The relation of the inhibitory effect to steps in the cycle of vaccinia virus multiplication is not known.

4. 5, 6-*Dichloro*-1-*β*-D-*ribofuranosylbenzimidazole* (*DRB*)

a. Inhibition of Influenza Virus Multiplication. This compound, designated DRB, was synthesized during a systematic investigation of the inhibitory activity of glycosides of benzimidazoles in relation to their chemical structure (Tamm *et al.*, 1954). It inhibits the multiplication of influenza B virus in the chorioallantoic membrane of the chick embryo *in vitro* (Tamm *et al.*, 1954).

In single-cycle multiplication experiments (Tamm and Tyrrell, 1954), the compound at 0.018 mg. per milliliter inhibited reproduction markedly when added at 1 or 2 hours but had little or no effect at 3 hours after inoculation. It also inhibited the production of soluble complement-fixing antigen, though less markedly than that of virus particles, when added at 0.5 hours, but not at 1 hour. Both as regards virus multiplication and production of soluble antigen, the later the compound was added during the latent period the smaller was the degree of inhibition. The latent period of influenza B virus in

the chorioallantoic membrane *in vitro* is probably somewhat less than 4 hours (Tamm and Tyrrell, 1954). The compound is most effective during about the first half of the latent period, and has little or no inhibitory activity during the last half of the period. Its inhibitory effect on the production of the soluble antigen is restricted to about the first eighth of the latent period.

DRB does not inactivate influenza viruses *in vitro*, has no effect on virus-erythrocyte interaction, does not interfere with adsorption of the virus by host tissue, and does not affect the release of new virus particles from the chorioallantoic membrane (Tamm *et al.*, 1954). At concentrations causing 99 % inhibition of influenza B virus multiplication it does not affect the oxygen uptake of the membrane nor does it diminish tissue proliferation in roller tube culture. At higher concentrations it retards cell proliferation but this effect is reversed on removal of the compound (Tamm *et al.*, 1954). When the concentration is increased sufficiently, reduction in oxygen uptake and both microscopic and macroscopic damage of the membrane occur (Tamm, 1956b).

Because the inhibition caused by DRB is produced only during the first half of the latent period and because it does not affect any other step in the process of infection, it seemed probable that it acts by interfering with metabolic processes involving ribofuranosides and that it might affect nucleic acid metabolism (Tamm *et al.*, 1954). This hypothesis was suggested by the close chemical relationships between α-ribazole, a moiety of vitamin B_{12}, adenosine, a constituent of ribonucleic acid, and DRB (Tamm *et al.*, 1954). Recently Tamm (1957) demonstrated that DRB does in fact interfere with the incorporation of adenosine-8-C^{14} into ribonucleic acid (RNA) of the chorioallantoic membrane. Moreover, Allfrey *et al.* (1957) showed that DRB greatly reduces the incorporation of orotic acid-C^{14} into nuclear RNA and seems to block the synthesis of RNA within the nucleus, no matter when it is added to the medium. The finding that DRB does not inhibit multiplication after the latent period suggests that inhibition of host cell RNA synthesis at this late stage is of no consequence in virus reproduction (Tamm, 1958).

Studies of the incorporation of C^{14}-L-alanine into the protein of the chorioallantoic membrane indicate that DRB has little if any effect on protein synthesis (Tamm, 1957). If nucleic acid precursors of influenza virus are synthesized in the virus-infected cell before protein precursors, as appears to occur with bacterial viruses (Hershey and Melechen, 1957), it may not be surprising that DRB inhibits multiplication only during the first part of the latent period (Tamm and Tyrrell, 1954).

The high inhibitory activity of β-D-ribofuranosides of benzimidazole, relative to influenza virus multiplication, depends not only on the number of

halogen atoms present in the benzenoid ring but also on the structure of the carbohydrate moiety (Tamm, 1956b; Tamm *et al.*, 1956). α-Linked ribofuranosides of halogenated benzimidazoles are much less inhibitory than the corresponding β-linked derivatives. For highest inhibitory activity against influenza virus, which contains RNA (Ada and Perry, 1954), the carbohydrate moiety must not only be ribose but also a ribofuranose in β-linkage (Tamm, 1958).

Not only is DRB a potent inhibitor of the multiplication of influenza B virus (LEE and MB strains) in the chorioallantoic membrane *in vitro*, but also it inhibits the multiplication of influenza A virus (PR8 and FM1 strains) to the same extent in this host tissue (Tamm *et al.*, 1954). In addition, it inhibits the multiplication of the LEE strain in the intact chick embryo and in mice without causing significant signs of toxicity in either host species (Tamm *et al.*, 1954). Moreover, DRB, as well as the corresponding trichloro compound (TRB), inhibit the reproduction of mumps virus in the allantoic sac of the chick embryo (Tamm, 1954b).

b. Inhibition of Poliovirus Multiplication. DRB inhibits the multiplication of poliovirus, type 2, in monkey kidney cells *in vitro* (Tamm and Nemes, 1957). In single-cycle multiplication experiments (Nemes and Tamm, 1958), the compound at 0.03 mg. per milliliter effectively inhibited reproduction when added at 2 hours, but had little or no effect when added at 4 hours. The latent period of poliovirus virus in monkey kidney cells *in vitro* is about 4 to 5 hours (Lwoff *et al.*, 1955; Fogh, 1955). The compound, therefore, is effective only in the early part of the latent period, and becomes much less effective as the later part of the period is approached.

DRB does not inactivate the infectivity of poliovirus *in vitro* (Tamm and Nemes, 1957) and probably does not affect attachment or penetration of the virus, for inhibition was no more marked when it was added with the virus than at 2 hours after (Nemes and Tamm, 1958).

Poliovirus contains RNA (Schwerdt and Schaffer, 1955), and the inhibition of RNA synthesis caused by DRB in monkey kidney cells (Nemes and Tamm, 1958) may be correlated with the inhibitory effect of the compound on the multiplication of the virus during the early part of the latent period. DRB has only a slight effect on protein synthesis by monkey kidney cells *in vitro* (Nemes and Tamm, 1958).

As with inhibitory activity against influenza virus, any departure from the β-D-ribofuranose structure in the carbohydrate moiety of gylcosides of chlorobenzimidazoles reduced inhibitory activity relative to poliovirus virus multiplication (Tamm and Nemes, 1957). Because of the similarity in the nucleic acids of the two viruses and the demonstration that DRB inhibits RNA sythesis in the host cells used for both agents, correspondence between the results was anticipated (Tamm and Nemes, 1957).

In contrast, the multiplication of vaccinia virus, which contains deoxyribonucleic acid (DNA) (Hoagland *et al.*, 1940; Peters and Stoeckenius, 1954), is not inhibited more effectively by β-D-ribofuranosides of chlorobenzimidazoles than by the halogenated benzimidazole without the ribose carbohydrate moiety (Tamm and Overman, 1957). Furthermore, it appears that DRB does not inhibit the synthesis of DNA in concentrations at which synthesis of RNA is markedly reduced (Allfrey, 1958). This suggests that relative susceptibility to inhibition by DRB may reflect the nature of the nucleic acid in the virus particle (Tamm and Nemes, 1957).

5. *p-Fluorophenylalanine (FPA)*

This compound inhibits the multiplication of poliovirus, type 3, in HeLa cell cultures *in vitro* (Ackermann *et al.*, 1954). In single-cycle multiplication experiments, 0.1 mg per milliliter of the compound completely suppressed multiplication of the agent when the inhibitor was added at 1 or 2 hours. It was much less effective when added at 3 hours and caused no inhibition when added at 4 or 5 hours. On the basis that the latent period of this virus in HeLa cells is about 4 hours (Ackermann *et al.*, 1954), the compound is most effective during the early part of the period and is ineffective during the last half of the interval.

FPA does not inactivate the infectivity of poliovirus *in vitro* (Ackermann *et al.*, 1954) and probably does not interfere with the initiation of the incipient stages of infection. The compound inhibited the multiplication of HeLa cells but did not destroy their viability, for a normal rate of cell multiplication was observed on removal of the inhibitor.

The inhibitory effect of FPA on poliovirus multiplication is completely reversed by phenylalanine if the amino acid is added within 6 hours, but not later, after inhibition has been induced (Ackermann *et al.*, 1954). These data suggest that some function of phenylalanine is inhibited by the metabolic antagonist, FPA, and that this function is required only in an early stage of the multiplication of the virus.

Although the compound effectively inhibited poliovirus multiplication, it did not prevent the cytopathogenic effect of the virus. It should be pointed out that, at the concentration used, the compound itself caused some alteration in the morphology of the host cells. Disintegration of infected cells proceeded at the ordinary rate, without any increase of the infectious agent (Ackermann *et al.*, 1954). This was taken to indicate that the process leading to virus multiplication and to cellular injury possess a significant degree of autonomy. These results are closely similar to those obtained earlier in inhibition experiments with proflavin and bacterial virus, T2 (Foster, 1948).

6. *Levo-γ-(o-chlorobenzyl)-δ-oxo-γ-phenyl Caproic Acid*

This compound, termed caprochlorone, inhibits the multiplication of influenza A virus in the de-embryonated egg (Liu *et al.*, 1957a). In single-cycle multiplication experiments (Liu *et al.*, 1957a), 2 mg. of the compound per egg prolonged the latent period and delayed multiplication when the compound was present from 0 to 1 hour, 2 to 4 hours, or 4 to 6 hours. Addition of the compound at a later time had relatively little inhibitory effect. The earlier the compound was added, the more pronounced was the prolongation of the latent period. As judged from the final yield of virus, the inhibition produced by a 1- or 2-hour exposure of the infected tissue to the compound was relatively slight. However, when the compound was added at 2 hours and not removed, the yield of virus was reduced to less than 1 % of control values. The inhibitory effect on the production of soluble complement-fixing antigen was less marked than on reproduction of the virus.

Caprochlorone does not inactivate the infectivity of the virus *in vitro*, does not affect adsorption of the agent by host cells, and does not reduce oxygen uptake of host tissue. The effect on infected tissue is not irreversible and, on removal of the compound, recovery of full capacity to support virus multiplication returns in 12 to 18 hours (Liu *et al.*, 1957a).

In the lung of the intact mouse, caprochlorone, when given by gavage, 3 to 4 mg. per dose, 3 times daily for 9 days, diminished the extent of pulmonary lesions, mortality, and amount of virus produced if the inoculum was small (Liu *et al.*, 1957b). The yield of virus was approximately 10 % of that in controls. The claim that the compound exerts a therapeutic effect *in vivo* (Liu *et al.*, 1957b) is mitigated by the fact that it was given at the time of inoculation and was effective only when about 10 MLD_{50} of virus was given. The compound is toxic; 6 mg. causes death of chick embryos within 24 hours (Liu *et al.*, 1957a). There is no indication as to the mechanism of action.

7. 5, 6-*Dichloro*-1-α-D-*arabinopyranosylbenzimidazole* (*DAB*)

This compound, designated DAB, was also synthesized during a systematic investigation of the inhibitory activity of glycosides of benzimidazoles in relation to their chemical structure (Tamm, 1956b). It inhibits the multiplication of poliovirus, type 2, in monkey kidney cells *in vitro* (Tamm and Nemes, 1957).

In single-cycle multiplication experiments (Nemes and Tamm, 1958) the compound at 0.34 mg. per milliliter markedly inhibited reproduction throughout the latent period. Its effect was more striking when DAB was added at 3 hours than at 5 hours but was still obvious beyond the end of the latent period. Like DRB described in an earlier section, DAB has no effect on the infectivity of poliovirus *in vitro* (Tamm and Nemes, 1957) and probably does not interfere with attachment or penetration of the virus.

DAB interferes with the synthesis of RNA in monkey kidney cells *in vitro* but, unlike DRB, it also markedly interferes with the synthesis of protein (Nemes and Tamm, 1958). These findings may be correlated with the longer interval during which the inhibitory activity of DAB is manifest.

The compound shows relatively low activity, on a molar basis, as an inhibitor of influenza virus multiplication (Tamm, 1956b) and is only slightly more potent as an inhibitor of poliovirus reproduction (Tamm and Nemes, 1957).

B. Résumé of Inhibition during Latent Period

As shown in Table I, the 7 substances that have been demonstrated to inhibit multiplication during the latent period have very little in common; 3 are derivatives of benzimidazole, one is an analog of methionine, one is a bacterial capsular polysaccharide, one is a derivative of phenyl caproic acid, and one is an analog of phenylalanine. Only one, *K. pneumoniae*, type B, capsular polysaccharide (Fr. B), has been shown to inhibit reproduction during the latent period in an intact mammalian host, the mouse. Five show such inhibitory activity either in host tissue, i.e., chorioallantoic membrane, or in host cells, i.e., monkey kidney or HeLa cells, *in vitro*; one, in the de-embryonated egg.

Only five viruses, i.e., pneumonia virus of mice (PVM), influenza A (IAV), influenza B (IBV), poliovirus, type 2 (Polio, 2), and poliovirus, type 3 (Polio, 3) have been shown to be inhibited during the latent period. Only one compound, i.e., a chloro-ribofuranosyl derivative of benzimidazole (DRB), inhibits two of these viruses during the latent period. Four compounds, i.e., 2, 5-dimethylbenzimidazole (MB), methoxinine, caprochlorone and a chloro-arabinopyranosyl derivative of benzimidazole (DAB), are inhibitory throughout the latent period; all but methoxinine are somewhat inhibitory for some time after this interval. Only 3 substances show inhibitory activity that is restricted to the early part of the latent period, i.e, *K. pneumoniae*, Type B, capsular polysaccharide (Fr. B), DRB, and FPA.

There are indications of the biochemical mechanism of inhibitory activity for only four compounds: (1) Methoxinine blocks some function of methionine metabolism; (2) DRB interferes with the synthesis of RNA; (3) FPA interferes with phenylalanine metabolism; (4) DAB interferes with the synthesis of both RNA and protein. If virus precursor RNA is synthesized early in the latent period of influenza and polioviruses, it might be expected that DRB would inhibit reproduction exclusively during the early part of the interval, as is the case in both instances.

C. Inhibition after Latent Period

The only substances that appear to have some inhibitory effect on multiplication after the latent period has been completed are certain of those which

also are inhibitory during the latent period. Each of these substances was considered in some detail in the preceding section.

TABLE I

INHIBITION OF MULTIPLICATION DURING LATENT PERIOD

Inhibitory		Virus	Host system	Inhibition in latent period	Probable mechanism of inhibition	Reference
Substance	Amount					
Friedländer polysaccharide (Fr. B)	0.1 mg.	PVM	Mouse lung	First 2/3	Unknown	Ginsberg and Horsfall, 1951b
2, 5-Dimethylbenzimidazole (MB)	0.38 mg./ml.	IBV	Chorioallantoic membrane *in vitro*	All +	Unknown	Tamm *et al.*, 1953a
Methoxinine	1.3 mg./ml.	IAV	Chorioallantoic membrane *in vitro*	All	Methionine metabolism	Ackermann and Maassab, 1954b
5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)	0.018 mg./ml.	IBV	Chorioallantoic membrane *in vitro*	First 1/2	RNA synthesis	Tamm and Tyrrell, 1954
5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)	0.03 mg./ml.	Polio, 2	Monkey kidney *in vitro*	First 1/2	RNA synthesis	Nemes and Tamm, 1958
Fluorophenylalanine (FPA)	0.1 mg./ml.	Polio, 3	HeLa cells *in vitro*	First 1/2	Phenylalanine metabolism	Ackermann *et al.*, 1954
Caprochlorone	2 mg.	IAV	De-embryonated egg	All +	Unknown	Liu *et al.*, 1957a
5, 6-Dichloro-1-α-D-arabinopyranosylbenzimidazole (DAB)	0.34 mg./ml.	Polio, 2	Monkey kidney *in vitro*	All +	RNA and protein synthesis	Nemes and Tamm, 1958

In the absence of studies on inhibition of virus multiplication in single infected cells, it is uncertain how much validity should be ascribed to results that point toward inhibitory effects after the expiration of the latent period. With animal viruses, inhibition studies have so far been done with either intact tissues or large populations of cells in culture. Purposeful synchronization of infection has been attempted only with influenza A virus by means of pretreatment of tissue with methoxinine (Ackermann and Maassab, 1954b). In other instances, there is the likelihood that various steps in the complex process occurred at different rates in different cells and that this asynchronism affected results in multicellular systems. It should be emphasized that the latent period is defined as the interval before any new infective virus particles appear. It is, therefore, a measure of the time required to produce new particles by the first cells to yield. This is a minimal rather than a mean value; later yielding cells obviously may have longer latent periods than those that first produce new particles.

2, 5-Dimethylbenzimidazole (MB) caused definite inhibition of multiplication of influenza B virus in single-cycle experiments, even when added at 4 or 5 hours after inoculation (Tamm *et al.*, 1953a; Tamm and Tyrrell, 1954). In the host tissue system used, the latent period of the virus is probably less than 4 hours (Tamm and Tyrrell, 1954). The compound appears therefore to have been inhibitory for at least one hour after the end of the latent period, even though the extent of the inhibition produced was not large.

Caprochlorone appears to have caused some inhibition of multiplication of influenza A virus in single-cycle experiments, when added 4 hours after inoculation (Liu *et al.*, 1957a). In the host tissue used, the latent period of the virus is almost certainly less than 4 hours (Liu *et al.*, 1957a). The amount of inhibition produced was appreciable.

A chloro-arabinopyranosyl derivative of benzimidazole (DAB) caused marked inhibition of multiplication of poliovirus, type 2, in single-cycle experiments, even when added 5 hours after inoculation (Nemes and Tamm, 1958). The latent period of the virus, in the host cell system used, is about 4 hours (Fogh, 1955).

Although the mechanism of inhibition is not known for MB or caprochlorone, there is information regarding it for DAB. Not only does the compound interfere with the synthesis of RNA but also it inhibits the synthesis of protein (Nemes and Tamm, 1958). As indicated in the preceding section, this activity contrasts sharply with that of DRB, which interferes primarily with RNA synthesis and is inhibitory only during the first half of the latent period (Nemes and Tamm, 1958).

IV. Inhibition of Release

In most instances studied, animal virus particles appear to be liberated or released from infected host cells at a relatively slow rate (Cairns, 1952;

Dulbecco and Vogt, 1954; Ackermann and Maassab, 1954a) and do not emerge suddenly, as at the burst of phage-producing bacteria. The mechanism of release is not fully understood but it seems clear that certain viruses escape from infected cells which remain morphologically intact. This occurs most obviously with influenza virus in tissues of the chick embryo, the system in which release has been most extensively studied (Henle, 1949; Fazekas de St. Groth and Cairns, 1952; Murphy and Bang, 1952; Morgan *et al.*, 1956).

The only compound that has been implicated in the inhibition of release is α-amino-*p*-methoxyphenylmethanesulfonic acid (Ackermann and Maassab, 1954a,b). This compound interferes with the release of newly formed influenza A virus particles from the chorioallantoic membrane *in vitro*. This effect is prevented by the receptor-destroying enzyme obtained from *V. cholerae* extracts. It has been proposed (Ackermann and Maassab, 1954b) that one function of the influenza virus enzyme is to facilitate the release of virus particles from the infected host cell. As indicated in an earlier section, the compound does not inhibit the multiplication of influenza virus during the latent period but does interfere with the initiation of infection (Ackermann and Maassab, 1954b).

The great bulk of substances reported to have an inhibitory effect on animal virus multiplication have not been studied in single-cycle multiplication experiments. Because of the lack of precise kinetic data, it is not feasible to assign their inhibitory activity to any stage or step in the process of reproduction. At present, therefore, they must be grouped together in an unsatisfactory category: substances that are inhibitory at undetermined stages of the multiplication cycle.

V. Chemical Structure and Inhibitory Activity

In recent reviews (Matthews and Smith, 1955; Hurst and Hull, 1956; Horsfall and Tamm, 1957; Tamm, 1958), almost all known inhibitory substances have been discussed in some detail. At the risk of some repetition, it seems desirable to consider the activity of certain arbitrarily selected groups of compounds of known structure for which there are some indications of the mechanism of their inhibitory effects.

A. Benzimidazoles and Derivatives

More is known of the chemical structure-inhibitory activity relationships with derivatives of benzimidazoles than with any other group of chemical compounds (Tamm, 1958). Beginning in 1952, Tamm and his co-workers undertook a systematic evaluation of the effects of a variety of substitutions in the benzimidazole molecule and measured inhibitory activity with a

number of viruses. In addition, they made closely correlated studies of the damaging effects of the compounds by various means in a number of host cell systems.

These comprehensive investigations culminated in the synthesis of compounds with greatly increased inhibitory potency which interfere with the multiplication of influenza, mumps, vaccinia, and polioviruses. Certain of the compounds developed are moderately selective and show high inhibitory activity without a corresponding increase in host cell damage (Tamm, 1958). As stated in an earlier section, one highly potent and selective compound, DRB, has been found to interfere with the synthesis of RNA in the chorioallantoic membrane *in vitro*; another, DAB, has been demonstrated to interfere with the synthesis of both RNA and protein in monkey kidney cells *in vitro*. These findings provide a basis for an understanding of the biochemical mechanisms of their inhibitory effects (Tamm, 1958).

Benzimidazole is structurally related to adenine and is found as a part of the vitamin B_{12} molecule (Tamm *et al.*, 1952). The compound was first shown to have an inhibitory effect on vaccinia virus multiplication in tissue culture (Thompson, 1947; Thompson *et al.*, 1950). Later it was found to inhibit the multiplication of Theiler's GD VII virus in tissue culture (Rafelson *et al.*, 1950) and poliovirus, type 2, in tissue culture (Brown, 1952). It had a relatively small effect on experimental poliomyelitis in monkeys and no inhibitory effect on the disease in mice (Brown *et al.*, 1953).

Systematic alteration of the structure of benzimidazole by substitution of alkyl radicals at various positions in one or both rings markedly affected inhibitory activity against influenza B virus multiplication in the chorioallantoic membrane *in vitro* (Tamm *et al.*, 1953b). Both the position and the nature of substituent groups appeared to be of decisive importance. The most marked increases in inhibitory activity were achieved by the most extensive substitution in either ring. Among the more potent compounds were the 2, 4, 5, 6, 7-pentamethyl, the 5, 6-diethyl, and the 2-ethyl-5-methyl derivatives, two of which caused 75 % inhibition at approximately 200 μM concentrations. Further extension of these studies showed that chloro-substituted benzimidazoles were 2 to 3 times more active than corresponding methyl derivatives (Tamm *et al.*, 1954).

Recognizing that the 5, 6-dimethylbenzimidazole moiety in vitamin B_{12} and the adenine and guanine moieties in nucleic acids are linked to pentoses Tamm *et al.* (1954) undertook the synthesis and evaluation of a series of N-glycosides of variously substituted benzimidazoles. It was found (Tamm *et al.*, 1954, 1956; Tamm, 1954b, 1956b) in all cases studied, that the β-D-ribofuranoside was more active than the corresponding halogenated benzimidazole not containing the ribofuranosyl moiety. With the β-D-ribofuranosides of 6 halogen derivatives, the type of halogen substituent in the

benzenoid ring was not especially important. However, conversion of certain methyl benzimidazoles, not containing halogen substituents, glycosides including ribofuranosides served to eliminate inhibitory activity.

The high activity of β-D-ribofuranosides depends not only on the number of halogen substituents in the benzenoid ring but also on the nature of the carbohydrate moiety. Conversion of a dichloro derivative to the ribopyranoside failed to increase activity and conversion to the arabino-, galacto-, or glucopyranoside caused a decrease in activity (Tamm *et al.*, 1954). Moreover, α-linked ribofuranosides were much less active than the corresponding β-linked derivatives. For the highest inhibitory activity against influenza virus, which contains RNA, the carbohydrate moiety must not only be ribose but ribofuranose in β-linkage (Tamm, 1958). The structure-activity relationships suggest that the most potent compounds interfere with RNA synthesis directly, not with the activities of vitamin B_{12}.

Similar conclusions have been reached in comparable studies with poliovirus (Tamm and Nemes, 1957), which also contains RNA. The most active inhibitors against this agent were β-linked ribofuranosides. Any departure from the β-D-ribofuranose structure in the carbohydrate moiety of the benzimidazole glycoside results in reduced inhibitory activity against both polioviruses and influenza viruses (Tamm, 1958).

In sharp contrast to the results secured with RNA-containing viruses were those obtained with vaccinia virus (Tamm and Overman, 1957), which contains DNA (Hoagland *et al.*, 1940; Peters and Stoeckenius, 1954). Conversion of chloro benzimidazoles to corresponding β-D-ribofuranosides did not increase inhibitory activity against this virus. Recently, Allfrey (1958) demonstrated that a β-D-ribofuranoside (DRB), discussed in an earlier section, which markedly interferes with RNA synthesis (Tamm, 1957; Allfrey *et al.*, 1957), does not inhibit the synthesis of DNA.

Although it seems clear that the effectiveness of β-D-ribofuranosides of chloro benzimidazoles as inhibitors of virus multiplication reflects the nature of the nucleic acid in the virus particle, it is becoming apparent that other factors, not yet fully understood, may also be important (Tamm, 1958).

In addition to the effects of glycosides of benzimidazoles on RNA synthesis, those of certain compounds on protein synthesis may contribute to inhibition of multiplication. As described in a preceding section, an α-D-arabinopyranoside of chloro benzimidazole (DAB), although not highly potent as an inhibitor of influenza or poliovirus, is effective throughout the whole of the latent period of the latter virus. This compound interferes with both RNA and protein synthesis (Nemes and Tamm, 1958).

1. *Selectivity of Benzimidazoles*

Quantitative estimation of the toxicity of compounds for host tissue or cells *in vitro*, through observation of macroscopic changes, reduced oxygen consumption, or microscopic abnormalities, provides a means for relating toxicity to inhibitory activity (Tamm, 1956b; Tamm and Nemes, 1957). The relation between the two activities has been referred to as selectivity (Tamm, 1955).

Marked differences have been found in the selectivity of various benzimidazole derivatives (Tamm, 1956b). With numerous compounds, toxicity for the chorioallantoic membrane *in vitro* and inhibitory activity relative to influenza virus multiplication vary independently. With chloro-ribofuranosyl derivatives, chloro substituents in the benzenoid ring increased inhibitory activity considerably more than toxicity.

Toxicity for monkey kidney cells *in vitro* and inhibitory activity against poliovirus multiplication show different relationships (Tamm and Nemes, 1957). The α-linked arabinopyranoside (DAB) was the most selective compound studied; β-linked ribofuranosides were no more selective than unsubstituted benzimidazole in this system.

B. Amino Acids and Derivatives

A number of natural amino acids and a variety of derivatives have been found to inhibit the multiplication of various animal viruses (Matthews and Smith, 1955; Hurst and Hull, 1956; Tamm, 1958). In relation to the mechanism of inhibitory activity, consideration of the derivatives appears to be more fruitful than discussion of results secured with the natural compounds.

β-2-Thienylalanine inhibits the multiplication of vaccinia virus in chick embryo tissue *in vitro* (Thompson and Wilkin, 1948) and poliovirus, type 2, in tissue culture (Brown, 1952). In both instances, phenylalanine counteracted the inhibitory effect.

DL-Methoxinine inhibits the multiplication of vaccinia virus in tissue culture (Thompson, 1947) and influenza A virus in the chorioallantoic membrane *in vitro* (Ackermann, 1951a). In the latter instance, L-methionine, but not D-methionine, annulled the inhibitory effect. At inhibitory concentration the compound did not affect the respiration of the membrane. As indicated in a preceding section, some function of methionine rather than its synthesis is inhibited by the analog (Ackermann, 1951a) and this function is completed before infective virus particles appear (Ackermann and Maassab, 1954b).

DL-Ethionine inhibits the multiplication of poliovirus, type 2, in brain tissue *in vitro* (Brown and Ackermann, 1951), and influenza virus in the chorioallantoic membrane *in vitro* (Ackermann, 1951a). In both instances, the inhibitory effect was prevented by methionine.

p-Fluorophenylalanine inhibits the multiplication of GD VII virus (Pearson *et al.*, 1952) and poliovirus, type 3, in HeLa cell *in vitro* (Ackermann *et al.*, 1954). In the latter instance, the inhibition was completely reversed by phenylalanine. *p*-Fluorophenylalanine may be regarded as an inhibitor of protein synthesis (Halvorson and Spiegelman, 1952). The compound inhibited the multiplication of HeLa cells at virus inhibitory concentration. Although it completely inhibited poliovirus virus multiplication, it did not prevent the cytopathogenic effect of the virus and the distintegration of infected cells proceeded at the usual rate.

The results obtained with amino acid analogs are compatible with the view that these compounds interfere with or alter protein synthesis. In most instances, the inhibition of virus multiplication produced by the analog is prevented or reversed by the appropriate natural amino acid.

C. Purine and Pyrimidine Analogs

Relatively few analogs of purine or pyrimidine bases have been found to act as effective inhibitors of animal virus multiplication (Matthews and Smith, 1955).

Certain halogenated purines inhibit the multiplication of vaccinia virus in tissue culture (Thompson *et al.*, 1950) but the inhibitory effect is not prevented by natural purines.

2, 6-Diaminopurine inhibits the multiplication of vaccinia virus in tissue culture (Thompson *et al.*, 1950), Russian spring-summer encephalitis virus in tissue culture (Friend, 1951), and poliovirus, type 2, in tissue culture (Brown, 1952). In all instances, the inhibition was partially reversed by adenine. The multiplication of a number of other animal viruses was unaffected by this purine analog.

5-Chlorouridine or 5-hydroxyuridine inhibit the multiplication of GD VII virus in mouse brain tissue culture (Rafelson *et al.*, 1951; Pearson *et al.*, 1956). In both instances uridine partially reverses the inhibition. Various other pyrimidine analogs have not shown inhibitory effects with a variety of animal viruses.

On the basis of extensive studies with plant and bacterial viruses (Matthews and Smith, 1955), it has been suggested that purine and pyrimidine analogs show inhibitory activity, because on incorporation into nucleic acids they render them biologically nonfunctional.

D. Compounds Affecting Oxidative Metabolism

Marked alterations in the energy-yielding metabolism of host cells regularly lead to reduction in their capacity to support virus multiplication. Because the process of reproduction of viruses appears to be intimately linked to the

metabolic activities of the virus-infected cell, it would be surprising if such results had not been secured.

The multiplication of influenza viruses in the chorioallantoic membrane *in vitro* requires oxygen (Ackermann, 1951b; Tamm, 1956b). Pentamidine (Eaton *et al.*, 1952), 2, 5-dimethylbenzimidazole (Tamm *et al.*, 1953a) and antimycin A (Ackermann and Francis, 1954), at concentrations which diminish the oxygen uptake of the membrane, inhibit the multiplication of influenza viruses. It appears that the inhibitory effect is secured only if the endogenous respiration of the membrane is diminished during the latent period.

DL-Ethionine, 2, 6-diaminopurine, benzimidazole and β-2-thienylalanine, at concentrations which markedly inhibit the respiration of HeLa cells *in vitro*, also inhibit the multiplication of poliovirus, type 1 (Gifford *et al.*, 1954). At lower concentrations which do not diminish oxygen uptake, these compounds have no inhibitory activity against this virus.

The synthesis of influenza virus appears to be dependent upon some oxidative reactions of the Krebs cycle (Ackermann, 1951b). Chemical inhibition of enzymes of the citric acid cycle by malonate or fluoroacetate inhibited influenza virus multiplication. Such inhibition was demonstrated in the lungs of mice (Ackermann, 1951c). Later work showed that fluoroacetate delayed but did not prevent the multiplication of influenza virus or pneumonia virus of mice in the mouse lung or influenza and mumps viruses in the allantoic sac (Mogabgab and Horsfall, 1952).

2, 4-Dinitrophenol stimulates tissue respiration but inhibits oxidative phosphorylation. The compound inhibits the multiplication of influenza virus in the chorioallantoic membrane *in vitro* (Eaton, 1952; Ackermann and Johnson, 1953); the degree of inhibition is correlated with the extent of stimulation of respiration and release of phosphate. It has been suggested that the energy required for virus synthesis derives from the oxidative phosphorylative activity of the host tissue.

VI. Modification of Cell Damage

The virus-infected cell is metabolically different from the uninfected host cell. Recent evidence bearing on the metabolic alterations have been summarized by Tamm (1958) and new data have appeared since (Boyer *et al.*, 1957; Maassab *et al.*, 1957; Levy *et al.*, 1957). In the majority of instances, the multiplication of animal viruses leads to the production of definite abnormalities in infected host cells. Whether the damage that occurs in the virus-infected cell results from the biosynthetic alterations that are required for the process of multiplication or from the products of the process is not yet clear (Horsfall and Tamm, 1957).

In marked contrast to the large amount of information that has been assembled relative to inhibition of virus multiplication, very little is known regarding modification of the host cell damage that is so commonly associated with multiplication. There is as yet no evidence that any inhibitor of a biosynthetic process will protect the virus-infected cell from the cytopathogenic effects of the agent (Tamm, 1958).

As indicated in an earlier section, *p*-fluorophenylalanine, although markedly inhibitory against the multiplication of poliovirus virus, had no modifying effect on the cytopathogenicity of the agent in HeLa cells (Ackermann *et al.*, 1954). Although a culture filtrate of *Penicillium stoloniferum*, designated M-8450, prevented the cytopathogenic effects of all three types of poliovirus on monkey testicular cells *in vitro* (Hull and Lavelle, 1953), the mechanism responsible for the modification remains unclear. It is not yet known whether the filtrate prevents infection, inhibits virus multiplication, or acts in some other manner.

A product from *Achromobacter xerosis*, designated Xerosin, has been shown to modify some secondary virus-induced lesions in the mouse lung but does not inhibit virus multiplication (Groupé and Dougherty, 1956; Ginsberg, 1955). There is no evidence that the substance decreases damage to the virus-infected cell per se.

VII. Current Status of Inhibition of Multiplication

Within the last few years many striking advances have been recorded regarding inhibition of multiplication of animal viruses. Many compounds of diverse nature have been discovered to possess inhibitory activity in greater or lesser degree. Procedures have been devised for precise measurement of inhibitory potency. A technique has been developed which permits the selection of favorable alterations in chemical structure. Through its use compounds of greatly enhanced inhibitory potency have been produced.

Some inhibitory compounds of known structure have been shown to interfere with biosynthetic processes involving large molecular substances, i.e., nucleic acids and proteins, in the host cell. In a few instances, it has been possible to correlate the inhibitory activity with the interfering effect on host cell biosynthetic systems. However, it has not yet been feasible to demonstrate that the synthesis of a virus precursor material per se is affected by any inhibitory compound.

All potent inhibitory compounds have been found to have some effect on host cell metabolism, whenever such an effect has been sought (Tamm, 1958). Because of the lack of knowledge of any specific chemical features of animal virus components, approaches to the inhibition of biosynthetic systems have

been developed at the level of reactions which probably are common to both virus and host cell. As Tamm (1958) has emphasized, there is now impressive evidence that the synthesis of virus materials in host cells takes place in the absence of a limiting virus membrane. This and the intimate dependence of virus synthesis on host cell metabolism raise an important question: Will it be possible to inhibit the production of virus precursor materials in the virus-infected cell without also interfering with the synthesis of essential host materials? Although the question cannot be answered decisively on present evidence, it seems possible that quantitative differences in biosynthetic conditions, rates, and requirements may permit the process of multiplication to be selectively inhibited. Some inhibitory compounds have, in fact, already shown a moderate degree of selectivity and give evidence of possessing encouraging quantitative differences in inhibitory activity and host cell toxicity.

Relatively few animal viruses have been studied intensively in inhibition experiments. Pneumonia virus of mice, vaccinia virus, influenza virus, and polioviruses have for various reasons been selected as model agents in most studies. All investigations bearing on inhibition during the latent period have been performed with one or another of these viruses. Even within this small group of viruses, inhibitory compounds have shown strikingly different effects. In numerous instances, compounds that are highly potent against the multiplication of one virus are considerably less active or are inactive against that of another. In only an occasional instance has it been feasible to suggest a basis for such differences in inhibitory activity.

The great majority of inhibitory compounds have been shown to be active in host cell systems *in vitro*. Only a small number of compounds so far have been demonstrated to inhibit virus multiplication in the intact animal host. Many inhibitory compounds possess such low activity on a weight basis that it has not been feasible to use them at the desired concentration in the intact host. Among the more potent compounds which show relatively high activity, only a few have manifested selectivity of a degree sufficient to permit their use in amounts that were not toxic for the animal host.

With very few exceptions, compounds that are potent inhibitors of virus multiplication have given disappointing results in chemotherapeutic experiments. Only in a single instance, i.e., pneumonia virus of mice, has it been possible with an inhibitory substance to modify an experimental virus disease in favor of the host after gross lesions have appeared. As was stated earlier, no inhibitory material has as yet been shown to be useful in the management of any naturally occurring virus disease of animals or man. Whether inhibitory compounds will be brought to a stage of development in which they will have useful applications is an unanswerable and probably not a very important question. Their quest has already opened large new vistas

bearing on the mechanism of virus multiplication—a biosynthetic capability which appears to be common to all living things.

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Chapter IX

Variation in Virulence in Relation to Adaptation to New Hosts

Frank Fenner and John Cairns

Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

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I. Introduction

Although there is an increasing trend toward the study of animal viruses and the cells they infect as systems of intrinsic biological interest, the science of animal virology has until recently been developed as a branch of medical science, by men concerned primarily with viruses as agents of disease. The essential prerequisite for the experimental investigation of an animal virus or of a virus disease of man or a domestic animal is the production in some laboratory host of recognizable signs of infection associated specifically with the virus in question.

Such a result may be observed the first time the virus is inoculated into an experimental host. For example, cowpox virus from natural human or bovine

infections produces characteristic lesions on the chorioallantoic membrane or in the rabbit skin, and neither the nature of the lesions nor the "efficiency of plating" for the passage host changes with serial passage in either of these hosts. In other cases minimal signs of infection are observed on first introduction of the virus into a new host, yet after serial passage, sometimes prolonged, a lethal infection is regularly produced, e.g., the adaptation of poliovirus and dengue virus to mice (Armstrong, 1939; Sabin and Schlesinger, 1945). During serial passage a process of "adaptation" of the virus to the new host is said to have occurred.

Once a virus has been adapted to an experimental host, the attention of many workers has turned to a frequent by-product of adaptation, namely, the coincident attenuation of the virus for its original host. Thus, after his experiences with the attenuation and exaltation of virulence of bacteria by passage in different animal hosts Pasteur turned his attention to rabies (Pasteur *et al.*, 1884) and found that by serial passage of this virus in rabbit brain it changes from "street" virus to "fixed" virus, and showed enhanced virulence for the rabbit, and reduced (but still high) virulence for the dog and man. The best-known examples of the production of an attenuated virus vaccine by adaptation to a new host tissue are the yellow fever vaccines. The "French neurotropic" strain was obtained by serial passage in the mouse brain (Peltier *et al.*, 1940) and the 17D vaccine during prolonged passage in tissue culture (Theiler and Smith, 1937).

In consequence of the practical requirements just outlined, there has grown up an enormous volume of literature concerned with the growth of animal viruses in different laboratory hosts—involving primary adaptations, secondary adaptations from one laboratory host to another or one organ or tissue of a particular host to another, and studies of the effect of such adaptations on the pathogenic capacity of the virus in its original host. Because of the complexity of the experimental material, studies on the adaptation of animal viruses to new hosts have so far yielded little basic information, but have given rise to a great volume of superficial observations. These, although invaluable at the practical level, reveal virtually nothing of the mechanism of adaptation.

For most people, maximum virulence of a virus, as a character, indicates that the strain possesses the maximum destructive power which can be associated with the multiplication of a virus of that sort. With bacterial viruses, where the host is a unicellular microorganism, virulence implies cell lysis. The animal host, however, consists of a vast number of individual cells differing widely in many properties. Some may be suited and others unsuited to support virus multiplication, some are of major and others of minor importance in the economy of the host. In animals, therefore, there is no such simple dichotomy and no general agreement as to how the word virulent can

be defined. A strain of influenza virus which is virulent for man is, in common parlance, either one that spreads rapidly and extensively through the population, or one that is characteristically associated with a severe morbidity and, for influenza, a relatively high death rate. These effects may obviously be due to widely different and potentially very complex mechanisms. In the same way a strain of poliovirus may be called virulent for man because it paralyzes a high proportion of those it infects, or because it kills a high proportion of those it paralyzes. These vague uses of the term virulence, sanctioned by convention, merely amount to calling any strain virulent if it has high destructive power of the type associated with that virus. There are obviously many grades of virulence, not the simple alternatives we see in bacterial viruses. This usage does emphasize the medically and socially important aspect of the virus diseases of man and his domestic animals, but it deals only with the end product of a multitude of unknown factors, and tells us nothing at all of the possible mechanisms involved. There are more sophisticated definitions, but not one of these can be framed so that it will cover the activity of all animal viruses.

With this definition of virulence, it is apparent that nearly all examples of adaptation of viruses to new hosts involve increase in virulence for the new host. The field under review therefore covers nearly all examples of alteration in the biological behavior of viruses. Two ways of treating the subject present themselves. One would be to catalogue all examples of variation in host spectrum among animal viruses. This might be useful if the literature were both interesting and as yet inadequately reviewed, but it would be dull, brutish, and long. Catalogues of examples of variation in animal viruses have already been prepared (Findlay, 1936, 1939).

The other procedure, which will in fact be followed, is to try to break down the process of "adaptation" into its component parts, starting with the simplest system, i.e., animal cells in suspension, which is analogous to the bacteria which serve as hosts for bacterial viruses. With this start it will be possible to progress steadily through systems of increasing complexity where adaptedness necessarily constitutes a more complex property, and a strain of virus may be "adapted" or "unadapted" for any one of several widely different reasons. Ultimately, we shall discuss the only available example where the natural evolution of virulence of virus in virgin territory has been studied. First, we shall discuss briefly what is known of the mechanisms of virus variation.

II. Mechanisms of Virus Variation

For all living creatures, natural selection copes with, and occasionally profits by, the instability of the genome. So it is, too, for viruses. Survival of

the line is, as we shall see in the last example of variation in virulence, not assisted by exceptionally great lethality; if the host is rapidly killed, the chance of successful transplantation of the virus to another host may not be high (Burnet, 1945).

Throughout this chapter we are dealing with the variation of virulence and the way in which discernibly new varieties of virus arise. Occasionally, evidence is mentioned which indicates that, during the change in character of a virus, one population of virus particles is being replaced by another. But the process of natural selection must be read into everything that follows. At all levels of complexity, the adaptation of a virus to a new situation must be pictured as the product of a single change or succession of changes, augmented by intense selection pressure. What is known of the manner in which these primary changes occur will now be discussed briefly, taking first of all evidence from the field of bacteriophage to provide a basis for what little is known in the case of the animal viruses.

A. In Bacterial Viruses

First, and simplest, variants may arise by a process of random mutation occurring during virus replication; in this case, variant clones are found in the virus populations yielded by individual cells (Luria, 1945, 1951). The distribution of such clone sizes among the yields of individual cells shows that the change is random rather than directed by the host cell.

The second process operates primarily on the host cell; variant clones are distributed randomly among virus populations yielded by large groups of host cells, but these clones arise from particular cells which themselves are variants in that they give rise (uniformly or not) to virus variants (Fredericq, 1950a,b). Although such host-induced changes are usually only phenotypic (Luria and Human, 1952; Bertani and Weigle, 1953), there is apparently one instance of the change being ultimately genotypic (Fredericq, 1950a,b). In many instances, this distinction between primarily virus and primarily host-induced variation disappears, for there is increasing evidence of genetic homogeny between certain bacterial viruses and their hosts (Stent, 1958).

Thirdly, genetic recombination may provide a mechanism whereby changes resulting from the operation of the first two mechanisms may be transferred from one virus to another differing in many aspects of its genetic constitution. It is conceivable that genetic recombination may play an important part in the ecology of bacterial viruses.

B. In Animal Viruses

Adequately documented examples from the field of animal viruses of the three processes described in the previous section are so rare as to be almost unique.

1. *Variation by Mutation*

Although random mutation is usually invoked as the basis for variation in animal viruses and, on this basis, mutation rates have been calculated (Burnet and Bull, 1943; Medill-Brown and Briody, 1955; Dulbecco and Vogt, 1958), in only one instance has there been any attempt to demonstrate the essential character of a mutational change—namely, its randomness (Dulbecco and Vogt, 1958). In this instance, the d to d^+ mutation of poliovirus, it was possible to show significant fluctuation in the number of mutants present in sister populations of virus grown in aliquots of one cell population. In other words, sister populations of virus show nonrandom variation in proportion of mutants if multiplication has occurred since they were separated, whereas the variation is, of course, random if there has been no multiplication in the interim.

2. *Host-Induced Variation*

There are at least two examples of what is probably host-induced variation of an animal virus. The first, and less conclusive, concerns the behavior of unadapted influenza A virus and Newcastle disease virus (NDV) in mouse brain; after small inocula of non-neurotropic influenza virus into mouse brain, there is a single cycle of virus production, the virus produced being infective for the allantois but apparently not for mouse brain in that multiplication there was confined to one cycle (Cairns, 1951, 1954). The second concerns the behavior of encephalomyocarditis virus in mouse brain; a variant of this virus, adapted to form plaques on sarcoma 180 cells, loses its capacity to form plaques on these cells if passed through a single cycle in mouse brain; this capacity is restored after a single cycle in Krebs 2 carcinoma cells (Sanders and Hoskins, 1958).

3. *Genetic Recombination*

The genetic importance of sex in higher organisms, as a means of producing novel combinations of characters and providing the opportunity for rapid spread of mutant characters through the gene pool, needs no emphasis. The very short generation time and enormous populations of viruses provide a much greater opportunity for the combined interplay of mutation (random or host-induced) and selection to serve as the major basis for evolution.

Except possibly with bacteriophages it seems to us unlikely that genetic recombination is of importance in the evolutionary history of viruses, but recent work has shown that it may be a useful method for producing novel combinations of virus characters in the laboratory. In a number of animal viruses only phenotypic mixing has been detected, i.e., the novel combination dissociates to the parental types on passage. Genetic recombination has been unequivocally demonstrated with influenza virus (Burnet, 1955) and with

vaccinia virus (Fenner and Comben, 1958). In both cases characters concerned with pathogenicity or virulence behaved in a complex manner (as would be expected from their nature) and cannot yet be analyzed physiologically or genetically. However, genetic recombination should eventually prove a powerful weapon for the analysis of these most obscure and complex aspects of viral behavior.

III. Animal Virus-Host Cell Systems

It was noted earlier that all known associations of viruses with animals are much more complex than the relation between a bacterial virus and its host. For analytical purposes, however, it is possible to construct simpler artificial systems or to utilize situations which do not involve the whole complex activity of the animal. We shall consider three types of virus-cell system—simple cell systems, analogous to the bacterial virus-bacterium system; structurally complex but relatively self-contained cell systems, such as the chorio-allantoic membrane of the chick embryo; and cell systems involving sequential infection of a variety of organs. As a special case of the latter we shall introduce sequential infection of individual animals, i.e., we shall extend our study to include some epidemiological facets of adaptation and virulence.

A. Simple Cell Systems

This group comprises those examples of infection of cells by viruses in which spread of infection from cell to cell is not limited by the nature of the system and merely demands that, after infection of a cell, the progeny virus is liberated from that cell in a form which can infect further cells. In this group fall all examples of infection of cells in fluid suspension and also those examples of infection of cells in sheets, where extension of infection from cell to cell can occur freely by way of the overlying fluid. As examples of adaptation and variation in such systems we will discuss the work done on the changes in influenza virus on adaptation to the embryonated egg (the O-D change), and on the variants of poliovirus in tissue culture. In neither of these cases is the problem of spread from cell to cell a deciding issue in the success of virus multiplication.

1. *The O-D Change of Influenza Virus*

This example of variation of an animal virus was the first to receive detailed attention from the point of view both of the nature of the character undergoing change and the manner in which the change occurred. On first isolation, influenza A strains were found to agglutinate human and guinea pig red cells more readily than fowl red cells (Burnet, 1942). On further

passage, either in the amnion at low dilution or in the allantois, the virus acquired the capacity to agglutinate fowl cells to as high a titer as human cells. This change, from the O ("original") to the D ("derivative") phase, is associated with a change in affinity for various hemagglutinin inhibitors (Stone, 1951) and in position in various receptor gradients (Burnet *et al.*, 1946a, 1949); it is also associated with a marked reduction in virulence for man (Burnet and Foley, 1940) and emergence of capacity to multiply in the allantois (Burnet and Bull, 1943).

However, it has been possible to maintain virus in the O phase through repeated passages in the amnion if each passage is initiated at "limit dilution" (Burnet and Bull, 1943). This shows that a mere history of prolonged multiplication in the amnion is not in itself a certain inducer of the change to D phase: for this to happen there has to be full opportunity for minority populations to supplant the original type. It is as if D phase virus arises relatively infrequently during multiplication in the amnion; although endowed with considerable survival advantages over O phase virus, D phase virus must seldom represent the majority type after a single passage in the amnion. These considerations led Burnet to propose that the O-D change represents a relatively rare mutation backed by intense selection pressure, rather than a host-induced change. As we shall see later, an exactly analogous argument has been proposed as evidence for the mutational nature of the attenuation of poliovirus in tissue culture (Sabin *et al.*, 1954).

Unfortunately, the O-D change, on more detailed examination, proved to be more complex than originally supposed. The dividing line between agglutinative behavior of O- and D phase is not completely clear-cut. Between the two extremes (no agglutination of fowl cells, even in the cold, and agglutination of fowl and human cells to equal titer) there occur intermediates which are not easily distinguished from mixtures (Burnet and Stone, 1945a). Continued passage of O phase virus at limit dilution in the amnion tends to exaggerate the O character of its agglutination, so that strains which showed slight agglutination of fowl cells when originally isolated, show no agglutination of fowl cells after prolonged passage in the amnion; perhaps it was because of this that the definition of O phase agglutination became progressively more stringent with the passage of time (Burnet and Bull, 1943; Burnet and Stone, 1945a; Burnet, 1950, 1951; Burnet *et al.*, 1949). At least one strain, IAN, has stabilized in the O phase on passage in the amnion so that D mutants are not readily obtained (Burnet *et al.*, 1949); there is at least one instance of conversion of D phase to O phase by prolonged passage in the amnion (Magill and Sugg, 1948). Further, D phase virus (or intermediate phase virus) does not necessarily possess survival advantages over O phase in the amnion, since the only experiment testing the behavior of artificial mixtures in the amnion gave inconclusive results (Burnet and Stone, 1945a).

In fact, the O-D change does not really lend itself to a detailed examination of the processes of mutation and selection, since there is no clear-cut procedure for assaying separately either O or D phase virus. Originally it was supposed that only D phase virus multiplies in the allantois, which therefore could be used as a specific host for assay of D phase virus. However, O phase virus has been shown to multiply in the allantois to a limited extent (Burnet and Stone, 1945b), so that there is no precise procedure for distinguishing mixtures of O and D phase virus from intermediates. In view of these difficulties it is clear that no accurate determination of the relative survival advantages of the various types can be made, and hence no estimate of mutation frequency is possible.

Lastly, the idea that agglutinative behavior bears any simple relation to the host range or past history of any particular strain is itself difficult to sustain. Influenza B strains are not in the O phase on first isolation (Burnet *et al.*, 1946b; Hirst, 1947a); indeed, on passage, the agglutinative behavior of B strains tends to proceed in the reverse direction (Ledinko and Perry, 1955). Most recent influenza A strains have on isolation shown qualities closer to D than to O phase. And we shall see, in the section on adaptation of influenza virus to mouse lung, that changes in hemagglutinating behavior, although often associated with the process of adaptation, are not an obligatory step in the change in host range but a secondary phenomenon which may bear little relation to the alteration in host range.

2. *Variation of Poliovirus in Tissue Culture*

The study of the *d* mutation by Dulbecco and Vogt (1958) has already been discussed in the section of mechanisms of variation. It remains in this section to deal with those cases where there is some relation between neuropathogenicity and behavior in tissue culture.

In general, prolonged passage in tissue culture results in a reduction of neuropathogenicity. This, although not invariably true, has been demonstrated repeatedly [Enders *et al.* (1952) and countless others since] during the search for attenuated strains suitable as vaccines. There is some evidence for the belief that this attenuation occurs by way of selection of spontaneous mutants which have survival advantage in tissue culture and, incidentally, a lowered neuropathogenicity. Thus, passage of Mahoney, Y-SK, and Leon in cynomolgus kidney tissue cultures results in conversion to attenuated variants only if large inocula are used for each passage; if passage is conducted at "limit dilution" the strain keeps its neuropathogenicity, presumably because one passage will seldom provide adequate opportunity for the attenuated mutants to become the majority type (Sabin *et al.*, 1954). This result is exactly analogous to the findings with the O-D change (Burnet and Bull, 1943). Alteration of neuropathogenicity to mice of a line of type 3

poliovirus has been shown to be influenced by the type of cell used for tissue cultures as well as the constitution and pH of the medium (Li, 1957). These studies, however, have so far not been conducted with pure clones of virus; consequently, what is potentially an extremely interesting situation has yet to be exploited. Similarly, it has been shown that cells which are incapable of yielding virus when infected in the host will proceed to develop virus if taken from the animal and grown *in vitro* (Kaplan, 1955). Thus, environment may have a marked effect on the potentiality for cells to support virus multiplication.

Other variants with reduced neuropathogenicity have been demonstrated to arise in tissue cultures. Such are the minute plaque variants of MEF-1 which appear in the presence of bovine serum (Takemori *et al.*, 1957), and the variants of several strains which are capable of multiplying in monkey kidney cells at 30°C. (Dubes and Chapin, 1956). In the latter case, it is tempting to believe that the lowered neuropathogenicity is a simple consequence of a development of marked heat sensitivity; certainly it was only in those cases where the mutant proved incapable of multiplying in monkey kidney cells at 37°C. that neuropathogenicity was lowered.

Where looked for, there has been ample evidence of inhomogeneity of various lines of poliovirus. Heat-inactivation curves show the concavity which would result from inhomogeneity; part of this is due to stable characters which breed true on further passage (Stanley *et al.*, 1956), and part is due to some phenotypic difference not apparently reflected in genotype (Pohjanpelto, 1958). In this respect, poliovirus, in its heat-stability, is like certain bacteriophages (Adams, 1953) and viruses of the influenza group (Jones, 1945; Goldman and Hanson, 1955).

Just as poliovirus can be shown to vary in its host range, so can certain lines of once-cloned cell lines be shown to contain subpopulations with varying susceptibility to poliovirus which breed true (Vogt and Dulbecco, 1958). The more resistant types have a very low probability of being infected on meeting a virus particle; hence, with such cells, it is possible to establish infected cultures in which cell and virus multiplication can apparently coexist indefinitely unless something disturbs the balance (Vogt and Dulbecco, 1958). Whether it would be possible to select for new virus types, capable of infecting these resistant cells, has not yet been determined; this step has been taken only in a rather more complex situation (Sabin, 1952). However, there is every reason to believe that, as for bacteria and bacteriophages, both host cell and virus enjoy variation in susceptibility and host-range, respectively. The mechanism of cell insusceptibility in this case is probably not dependent upon the absence of adsorption (Vogt and Dulbecco, 1958), and so is not comparable to most examples of bacterial mutation to phage resistance.

3. *Various Examples of Coexistence of Virus and Cell*

At various times, instances of the coexistence of virus and cell multiplication have been demonstrated. These are of relevance here in that they represent a distinct type of interrelation of virus and cell which could be taken to epitomize one type of avirulence.

Production of vaccinia virus in roller tube cultures of fibroblasts was suggested to occur without preventing continued multiplication of the infected cells (Feller *et al.*, 1940). The peaceful existence in *Drosophila* of the virus of CO_2-sensitivity and its transmission through the egg is another example (L'Héritier, 1958), as must be all those cases where there is transovarial transmission in arthropods of viruses pathogenic for some other host (Fukushi, 1933; Syverton and Berry, 1941; Florio and Stewart, 1947; Black, 1950). Indeed, it has yet to be shown that the multiplication of any of the arthropod-borne viruses in their vectors is associated with any cell destruction.

None of these cases was amenable to the detailed quantitative investigation which is necessary in order to prove that liberation of virus by the cell is not necessarily associated with cell death. The establishment of a carrier state in tissue culture for poliovirus and NDV has recently been demonstrated (Ackermann, 1957; Cieciura *et al.*, 1957) and in these cases it should be possible to demonstrate nonlethal virus liberation, if it happens. So far, only for the infection of chicken fibroblasts by Rous sarcoma virus has it been demonstrated that a cell may liberate virus and then give rise to a relatively permanent line of multiplying cells, each of which liberates more virus (Temin, 1958).

These examples, some clear-cut, some merely suggestive, demonstrate the difficulties inherent in any definition of virulence. Rous sarcoma virus achieves its virulence for chickens by way of a relation with its host cell that represents an extreme variety of avirulent infection.

B. Structurally Complex Systems

In none of the examples of virus infection mentioned so far has there been the need to consider the manner of spread of virus infection from cell to cell. This great simplification was justified either because the example concerned only the immediate effects of infection (i.e., the first cycle of multiplication) or because the example concerned a host system in which it was reasonable to assume that there was no limitation in the spread of infection from cell to cell. However, when we consider that animals are characteristically multicellular organisms, whose component cells are organized in highly ordered groups often preserved behind a succession of defensive ramparts, it becomes obvious that animal viruses must possess well-ordered powers of infection if they are to spread successfully among the cells of their hosts.

Even in simple systems, the spread of infection is occasionally seen to be limited. Thus, varicella virus infection spreads among cells in tissue culture only by direct contact between cells and serial passage can be effected only with ground-up cells, not with the fluid supernatant (Weller, 1953).

Apart from those numerous instance (dealt with in the next section) where the natural course of infection demands the surmounting of successive barriers, there is ample evidence that the existence of an ordered organization of the host cells into a tissue may impose certain limitations upon the spread of infection. Thus, increasing the area involved by the inoculum by adding hyaluronidase, increases both the infectivity and the size of the resulting lesions of vaccinia virus in the rabbit skin, as well as increasing the probability of subsequent generalized infection (Duran-Reynals, 1929, 1933; Sprunt, 1941); this effect of hyaluronidase is seen also with herpes and vesicular stomatitis infection of rabbit skin (Hoffman, 1931). An enhanced response was found with Borna disease infection of rabbit brain (Hoffman, 1931), and with herpes infection of mouse brain (Levaditi *et al.*, 1949). Presumably in these infections the spread of virus from cell to cell is limited under normal conditions. Since, in the same tissues, hyaluronidase had no discernible effect upon the progression of infection by rabies, St. Louis encephalitis, or Lansing poliovirus, the infection produced by these viruses does not suffer the same limitations (Levaditi *et al.*, 1949).

Perhaps paralleling these observations are those examples in which the severity of a virus infection is increased by the presence of nonspecific irritants (Jones, 1950; Findlay and Howard, 1950a) or by coexistent infection by some quite unrelated virus (Lépine and Marcenac, 1948; Findlay and Howard, 1950b).

All examples of the production of localized lesions, whether in a two-dimensional structure, such as the monolayer overlaid by agar (Dulbecco, 1952), or a three-dimensional structure, such as the liver (Marchal, 1930), provide evidence for limitation of spread—spread either of the infecting virus particles or, in the case of the neoplastic viruses, of the primarily infected cells (Keogh, 1938). Histological studies have provided evidence for the slowness of spread of certain virus infections in the nervous system where others spread rapidly, and this has been demonstrated also by direct assay of the virus content of various parts of brain (Webster and Fite, 1934; Sabin and Olitsky, 1937; King, 1939).

Unfortunately, although this subject of the spread of virus infection through organized systems of cells may loom large in the future when knowledge of virus infection in simple systems has to be applied to complex hosts, available information at the moment amounts merely to demonstrating that the subject exists. Despite this lack, we shall now discuss certain examples of the variation of viruses infecting complex tissues under their own separate

heading as if the exact limitations imposed by each situation were precisely known.

1. *Pock Variants of the Poxviruses*

Infection of the chorion by the poxviruses results in a mixture of cellular proliferation, infiltration, and necrosis, involving both ectodermal and mesodermal cells (Burnet, 1938). The appearance of the pocks is therefore an index not only of the response of the infected cells but also the extent of spread of the infection and the reaction of the host animal—more complex properties than those that control, say, the morphology of the plaques produced by poliovirus in tissue culture. However, like plaques in tissue culture (Dulbecco and Vogt, 1954), each pock must be the result of infection by a single virus particle, since the number formed is proportional to the inoculum size (Burnet, 1936; Keogh, 1936; and many others since). For this reason the pock-producing viruses make a suitable group in which to study virus variation.

Cowpox virus (Downie and Haddock, 1952) and neurovaccinia (Fenner, 1958a) both give rise to white pock variants. These arise with a frequency of 10^{-2} to 10^{-4}, are stable on further passage, and presumably owe their difference from the normal hemorrhagic necrotic pock to a decreased tendency to invade underlying blood vessels and an increased tendency to cause cell infiltration. In both cases, attempts to demonstrate significant fluctuation in the frequency of white variants in sister populations of virus particles have failed, but it is not certain whether the observed absence of significant fluctuation is itself significant evidence that the change is not due to random mutation (Fenner, unpublished). Neither change appears to be host-induced in that the frequency of white variants is independent of the source of virus (pock on chorioallantoic membrane or nodule on rabbit skin) (Fenner, unpublished). In view of the exceptionally high frequency of the change (up to 10^{-2} for some strains) compared to all examples of mutation in other viruses, the possibility must be considered that, as in the case of the *r* mutation in the T-even bacteriophages, it may be the common phenotypic expression of a large number of different genotypes. Whether the wild type can be obtained from crosses between different white variants has yet to be determined.

Associated with this change in pock morphology there is a change in virulence for other hosts (Downie and Haddock, 1952; van Tongeren, 1952). The white variants produce less severe reactions in both rabbits and mice. However, the correlation between the pock morphology of the poxviruses and their virulence is not complete, for some strains of vaccinia and cowpox which produce hemorrhagic pocks have low neuropathogenicity for mice and rabbits, and some which produce white pocks are highly pathogenic.

Pock variants of herpes (Wildy, 1955) and myxoma viruses (Fenner and Marshall, 1957) have also been described. In both cases strains producing small pocks were usually less virulent for other hosts (mouse and rabbit) but the correlation was not complete, as the Californian strains of myxoma virus, though producing very small pocks, were highly virulent for rabbits.

2. *Adaptation of Influenza Virus to Mouse and Hamster Lung*

Many strains of influenza virus have been adapted by passage to produce fatal pneumonia in mice. The ease with which this process occurs varies greatly from strain to strain; some are apparently in the adapted state on first isolation from man (Francis and Magill, 1937; Clampit and Gordon, 1937), some are adapted readily, some become adapted only if special procedures are employed to lower the resistance of the mice (Jones, 1950), and some have so far defied all attempts. There is some evidence that adaptation occurs more rapidly if preceded by way of a few passages in ferrets (Andrewes *et al.*, 1935), and less readily if preceded by prolonged passage in the allantois (Smith *et al.*, 1951; Ledinko and Perry, 1955). Because most strains of influenza virus were not, in their natural state, adapted to any of the convenient laboratory hosts available in the 1930's, a large volume of literature has sprung up on the mechanism of adaptation to mouse (or hamster) lung and the nature of unadaptedness. The nature of the tissue being infected is more complex than any so far considered. Perhaps because of this the findings are, in places, singularly difficult to interpret.

Once again, the process of virus variation is seen to operate by way of an upsurgence of minority types. Selection of the majority type by passage at limit dilution in the allantois, between each passage in lungs, prevents the process of adaptation (Davenport, 1951). Also, as in the case of the O-D change, adaptation to mouse lung is associated with changes in *in vitro* behavior; thus, there may be a reduction in capacity to agglutinate fowl cells (Hirst, 1947b; Friedewald and Hook, 1948), change in position in the fowl cell receptor gradient (Ledinko, 1956), alteration in enzyme action on and sensitivity of hemagglutinin to ovomucin (Ledinko, 1956), mouse lung inhibitor (Davenport, 1952), and the β-inhibitor of mouse and ox serum (Chu, 1951; Smith *et al.*, 1951; Brans *et al.*, 1953; Briody *et al.*, 1955) and sheep salivary gland mucoid (Ledinko, 1955). Some of these changes in *in vitro* behavior have been shown to apply to an increasing proportion of the virus particles as adaptation progresses during passage; indeed, an association can be demonstrated, at any stage in adaptation, between the *in vitro* behavior of clones isolated from the lungs and their degree of virulence (Ledinko, 1956). Just as an increase in virulence seems to be associated with insensitivity to certain inhibitors, so the converse seems to be true; from a mouse-adapted strain there was isolated, by chance, a variant which was

earlier in the fowl cell gradient, had reduced enzymatic action against ovomucin, and was less virulent to mice (Isaacs and Edney, 1950).

This, then, is probably the most liberally documented instance of variation in virulence of a virus being associated with changes in *in vitro* behavior. There is, however, evidence that the association is not always perfect. Variants with extreme insensitivity to inhibitors may be obtained by passage of virus in eggs in the presence of bovine serum inhibitor; these variants, which arise with a frequency of about 10^{-8} (Medill-Brown and Briody, 1955) are not, however, fully adapted to mice (Chu, 1951). Conversely, during the process of adaptation to mice, clones of virus may be isolated which apparently are fully adapted to mouse lung and yet almost unaltered in their *in vitro* properties (Ledinko, 1956).

Unfortunately, there is more conflict of opinion over the nature of the change in *in vivo* behavior during adaptation; indeed, the conclusion seems inescapable that not all unadapted strains are unadapted for the same reason. Much of this difficulty is due to the exceedingly complex behavior of unadapted virus on first introduction to the mouse lung. Inoculation of large amounts of unadapted virus into mice results in extensive lung consolidation; on subinoculation of a suspension of these consolidated lungs little or no lung lesions result, and it is only after several more such passages—usually unaccompanied by any lung lesions and therefore constituting "blind" passages—that lung lesions reappear and become a regular feature of each passage (Anderson and Burnet, 1947; Sugg, 1949). In general, it seems that all unadapted strains are capable of multiplying in mouse lung at the first passage (Burnet and Stone, 1945b; Hirst, 1947b; Wang, 1948; Sugg, 1950; Davenport and Francis, 1951; Ginsberg, 1953a; Ledinko and Perry, 1955; Ledinko, 1956). [There has been only one suggestion that lesions may be produced in the absence of virus multiplication, and this example, the case of the related virus, Newcastle disease virus, is open to very considerable objection (Ginsberg, 1951)]. There is no doubt however that, in terms of hemagglutinin titer or infectivity for the allantois, unadapted strains produce lung consolidation and death much less readily than adapted strains. Part of this difference is probably due to the fact that, in terms of capacity to initiate multiplication, unadapted strains are about one hundred times less infective for mouse lung than adapted strains (Ginsberg, 1953b). But most of the difference in pathogenicity of unadapted and adapted strains almost certainly depends on the difference in their behavior once multiplication has been initiated.

Studies on the relative multiplication rates and final yields of adapted and unadapted strains in mouse (or hamster) lung have given conflicting results. In some cases, there is a clear increase in the rate of multiplication with adaptation (Wang, 1948; Ledinko, 1956), in some cases, not (Davenport and

Francis, 1951). In some cases, there is clearly an increase in the final maximum titer of virus in the lungs (Andrewes and Smith, 1937; Anderson and Burnet, 1947; Hirst, 1947b; Friedewald and Hook, 1948; Wang, 1948; Sugg, 1949, 1950; Davenport and Francis, 1951; Briody and Cassel, 1955; Ledinko and Perry, 1955; Ledinko, 1956), and in some cases, not (Hirst, 1947b; Davenport, 1952); and in some cases the final yield alters only in that it has a higher infectivity for the allantois in relation to its hemagglutinin content (Ledinko and Perry, 1955) or for mice in relation to its allantoic infectivity (Sugg, 1949; Ginsberg, 1953b). In many cases, too, there was evidence that the final yields of infective virus were high in the first one or two passages, passed through a minimum, and then rose again as the virus became fully adapted (Anderson and Burnet, 1947; Hirst, 1947b; Friedewald and Hook, 1948; Ledinko and Perry, 1955; Ledinko, 1956); this effect was seen even when each passage employed the same dose of virus in terms of hemagglutinin (Ledinko, 1956) and, at least in one instance, was associated with marked formation of incomplete virus in the first few passages but not in later passages (Ledinko and Perry, 1955). All these conflicting reports show that unadapted strains are not always unadapted for the same reason. Some show low multiplication rate, some produce low final yields, some mainly produce incomplete virus, and some (probably most) show a combination of these defects.

Part of the complexity of the situation may be a manifestation of the complexity of the mouse lung as a host for virus multiplication. First, although the system can for many purposes be regarded as a simple sheet of susceptible cells capable of adsorbing huge quantities of inoculated virus (Fazekas de St. Groth, 1950), there is definite evidence, even in the case of fully adapted strains, that virus multiplication fully exploits the system only after very large inocula. Thus, intranasal instillation of saline, a day or two after inoculation of virus, greatly increases the size of the lesions, so that mice die which would otherwise survive (Straub, 1940; Taylor, 1941); also, the fact that circumscribed lesions are produced by sublethal inocula of adapted strains implies that the system is not normally fully exploited. The second major complication to the interpretation of events is, of course, that the effect of antibody production can be demonstrated from about the third day (Donnelly, 1951). The effect of these two factors is that part of the change to the adapted state could, on occasion, represent an increase in capacity to spread through the system or a decrease in speed of evoking antibody formation; either of these changes could be manifest simply as an increase in final yield, without necessarily any alteration in infectivity for mouse lung or multiplication rate. Indeed, there is some evidence that unadaptedness to mouse lung may represent a very complex defect, for the process of adaptation is markedly hastened either by keeping the inoculated mice at

low temperature (Sulkin, 1945; Briody *et al.*, 1953) or by combining the virus inoculum with various nonspecific irritants (Jones, 1950).

C. Systems Involving Sequence

We have discussed variation and virulence in those systems where either one type of cell or one tissue is being infected. There remain those situations where a definite sequence of tissues is infected as the virus progresses toward that final stage by which it is regarded as virulent or not.

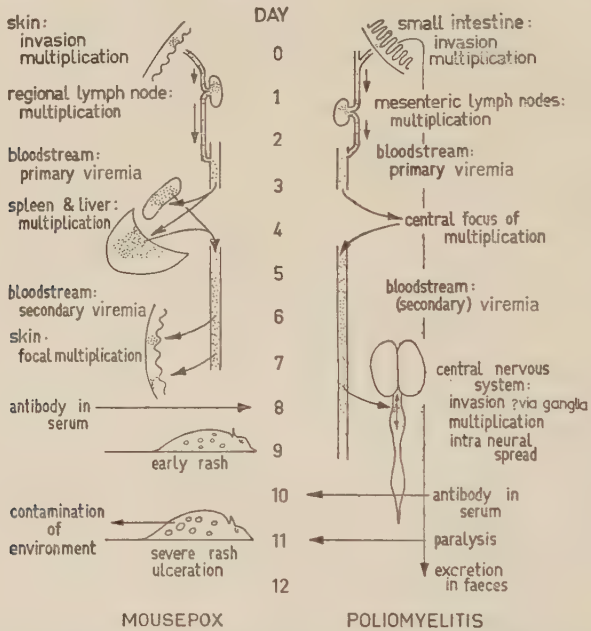

FIG. 1. Schematic diagram showing the mode of spread of mousepox virus and poliovirus in the host after infection by natural routes (modified from Fenner, 1956).

In a great many infectious diseases there is a stage of initial infection (which may be symptomless), a relatively long incubation period, and then a stage of generalization with toxic symptoms and symptomatic involvement of some particular organs or tissues, such as the skin (the exanthemata), the central nervous system (the encephalitides and poliomyelitis), or certain glands (mumps). In such diseases, the symptoms (and hence the assessment of virulence) do not depend on multiplication of the virus at the site of entry but upon multiplication at a number of distant sites.

Mousepox (infectious ectromelia of mice) has provided a model for a study of a sequential infection culminating in a rash (Fenner, 1948a). The scheme proposed, and its later extension to cover poliomyelitis (Fenner, 1956) are shown in Fig. 1.

Generalizing very broadly from this model, it can be said that in such virus infections several steps must occur in sequence: (1) Initial multiplication at the site of entry—this may eventually produce an apparent lesion, like the primary lesion in mousepox, or it may be inapparent. Sometimes multiplication of this type on a mucous surface (respiratory tract or gut) may produce free virus which can infect others. (2) At an interval after implantation, which may be short or not, virus enters the lymphatics. The local lymph node acts as a barrier to further spread, and a further process of virus multiplication must occur in the lymph node if the infection is to become generalized. (3) Once the lymph node barrier is surmounted, the virus has immediate access to the blood stream, and a wide variety of different sites of multiplication is available. Important among these are the vascular endothelial cells and the scavenger cells of the reticuloendothelial system (in the spleen, bone marrow, lymph nodes, and liver). The low entry rate of virus into the blood stream during this primary viremia, and the abundance of cells susceptible to infection ensure that little free virus can be recovered from the blood at this stage. (4) Progressive multiplication in the cells just entered, perhaps with further distribution of virus via the circulation, leads eventually to frank viremia. By the time this stage is reached changes have occurred in the host reaction due to antibody production (both sensitization, in its broadest sense, and serum antibodies being involved). (5) This combination of factors ushers in the next stage of the disease, i.e., localization and multiplication at the "secondary" sites (skin, brain, etc.) leading to the various typical syndromes of the generalized infection. Especially in the case of the central nervous system, there are barriers which may prevent this final step, perhaps in the vast majority of cases of infection.

Such a definite series of obligatory steps, each involving different types of cells, allows ample scope for different final results (i.e., different degrees of virulence) to be due to any combination of a number of variations in virus or host. Some virus strains may be avirulent because they merely have a low ability to multiply in the various tissues involved; others may be defective at some particular point in the sequence. The following examples of virus infections in systems involving sequence provide several instances of both types of defect.

1. *Mousepox*

Some of the less virulent variants of viruses which cause severe generalized diseases go through exactly the same sequence of events as the virulent strains, and are not "blocked" at any stage of the sequence. This is true of two attenuated strains of ectromelia virus, the "Hampstead egg" strain (Fenner, 1948b), and a strain passaged in tissue culture by Dr. G. Ruckle (Pittsburgh strain). Comparison of the growth of Moscow and Hampstead egg strain in various organs (Fenner, 1948b) showed that virus was detected

in the different organs on the same day with each strain, but that the titer rose more slowly and to a lower level in infections with the Hampstead strain. No detailed investigations have been made with the even less virulent Pittsburgh strain, but the virus certainly multiplies in the liver and spleen after peripheral inoculation and produces a slight rash in some mice.

2. *Yellow Fever*

The pathogenesis of yellow fever has been studied in rhesus monkeys by Theiler (1951). He found that, after intradermal inoculation of a moderately virulent strain, no multiplication of virus could be demonstrated in the skin but early multiplication occurred in the local lymph node. This was followed by invasion of the blood stream and then multiplication throughout the recticuloendothelial system (lymph nodes, spleen, and bone marrow) and, finally, by a stage of multiplication in the parenchymal cells of the liver, in the adrenal gland, kidney, and elsewhere. The attenuated 17D strain, on the other hand, multiplied in the local lymph node, invaded the blood, and was then found in the general lymphoid tissue and bone marrow, but to much lower titers than was the case with more virulent strains. Further, only occasionally was 17D found in the liver and then only in trace amounts. The attenuated strain thus multiplied to a lower titer in all sites and usually failed to infect the vital target site of virulent yellow fever virus, the liver. Strains intermediate in virulence between the 17D and the highly virulent Asibi strain showed intermediate levels of multiplication in the lymphoid and reticuloendothelial tissues, and in the liver.

A further effect of attenuation of yellow fever virus and other members of this group of arthropod-borne encephalitides is to lower the efficiency of their transmission by mosquitoes (Whitman, 1939; Hammon and Reeves, 1943). In part this operates merely by the reduction in the titer of circulating virus in infected animals, and in part by some specific inability of mosquitoes, even when infected, to transmit the attenuated virus (Whitman, 1939).

For viruses of this group, therefore, the stages comprising the whole sequence of infection includes those taking place in the vector—namely, infection and penetration of the gut wall, circulation in the hemolymph and localization in the salivary glands, and finally multiplication in the salivary glands and excretion in the saliva at the time of feeding. There are at least two instances of transmission being blocked by failure of the virus to surmount one of the barriers in the vector (Merrill and Tenbroeck, 1935; McLean, 1955).

3. *Poliomyelitis*

Poliomyelitis shows perhaps the most complex series of events between initial infection and the final production of symptoms. Figure 1 is probably an oversimplification, but it illustrates the existence of at least four stages—

occurring in the intestinal wall, the local lymph nodes, some central extraneural focus, and the central nervous system. Within the central nervous system there may be further barriers to the spread of virus, for histological examination of orally infected monkeys and chimpanzees has shown that asymptomatic infection of the nervous system may occur in some animals, whereas in others there may be extensive spread through the spinal cord and the brain.

Sabin's extensive studies on variation in the neuropathogenicity of the polioviruses show clearly the great complexity of this property. The impossibility of subjecting it to adequate genetic analysis at the present time is further exemplified by the observation that at least three different genetic factors are involved in the determination of neuropathogenicity (Vogt *et al.*, 1957).

Variants of poliovirus have been described which illustrate the failure of virus to multiply in one or other tissue of the sequence illustrated in Fig. 1. First, a high degree of neuropathogenicity of intracerebral inoculation may be dissociated from the ability to establish infection by feeding (Melnick, 1951; Sabin, 1956). Second, variants which are able to multiply in the alimentary mucosa (and provoke antibody formation) may fail to be immunogenic after intramuscular inoculation, i.e., such variants may fail to multiply in lymphoid tissue (Sabin, 1955a). Third, variants incapable of producing either paralysis or lesions after inoculation of large doses into the lumbar cord of chimpanzees will nevertheless multiply extensively in their alimentary tract (Sabin, 1955b,c).

During the last few years it has become apparent that the polioviruses are members of a large group of viruses which normally parasitize the enteric tract of man—a group now designated as the "enteroviruses." From the point of view of survival in nature, all that is required of such viruses is that they should be able to infect the cells of the alimentary tract and be excreted in the feces; this appears to be the limit of the activity of most members of the group. The aim of those who seek attenuated variants of the polioviruses for use as oral vaccines is essentially to obtain virus strains with the limited invasive power of most enteroviruses but the same antigenic constitution as those which occasionally invade the central nervous system of man. The major difficulty appears to be the occasional acquisition by attenuated variants of some degree of neuropathogenicity after multiplication in the intestinal tract of man (Sabin, 1955c; Dick and Dane, 1957). The suggestion made by Dick and Dane that, to counteract this risk, the virus should not be transmissible from vaccinated to nonvaccinated people may well be incompatible with the requirement that the oral vaccine should be immunogenic.

It is perhaps relevant that involvement of the central nervous system is of no evolutionary significance for most viruses, whereas in the exanthemata

transmission is dependent upon the virus produced at the final sites of multiplication (the skin and mucous membranes). Whether a virus is able to invade the nervous system is, possibly with the exception of rabies virus and a few others, immaterial to its survival: the ready multiplication of many viruses, when implanted directly into the brain, contrasts sharply with the rare occurrence of natural infection of the central nervous system.

The next section illustrates the effect of this further restriction on the selection of variants of different virulence, i.e., the necessity for efficient transmission between different host animals.

4. *Myxomatosis*

Infectious myxomatosis, in laboratory rabbits, is another generalized infection involving a sequential invasion of skin, lymph nodes, and vascular endothelial cells, and culminating in a widespread secondary rash (Fenner and Woodroofe, 1953).

Myxoma virus originated in South America, where it is enzootic in the local wild rabbit (*Sylvilagus braziliensis*). In this host it usually produces only a single localized skin tumor, and is transmitted mechanically by mosquitoes (Aragão, 1943). However, minute doses of myxoma virus, if transferred directly from the skin lesion of a *Sylvilagus* rabbit to the skin of an *Oryctolagus* rabbit, cause a very severe generalized disease, which is almost invariably lethal.

The deliberate introduction of myxomatosis into populations of wild *Oryctolagus* rabbits in Australia in 1950 (Ratcliffe *et al.*, 1952) and in Europe in 1952 (Radot and Lépine, 1953) provided an opportunity to see what changes in virulence would occur when a lethal generalized infection was introduced into virgin populations. In the present context it allows us to introduce a further factor into the sequence of events we have been discussing, and one of major importance in natural infections, namely, transmission from one animal in a population to another. Thus we may compare the simplest situation in animal viruses (transfer from one individual cell to another through a fluid menstruum) with one of the most complex (transfer from one mammal to another by an intermediate vector, with a complex sequence of events necessarily occurring in each mammalian host before the virus is available for transfer to another mammal).

With myxomatosis it is possible to use either the mortality rate or survival time after infection as a direct measure of virulence (Fenner and Marshall, 1957). The reverse procedure, namely, challenge of rabbits from areas with different histories of exposure to myxomatosis with one known strain of virus, made it possible to observe changes in the genetic resistance of wild rabbit populations (Marshall and Fenner, 1958).

In Australia and in France, myxomatosis is predominantly a summer mosquito-borne disease; in Britain, there is no marked seasonal incidence and the rabbit flea appears to be the important vector. The virus strains originally introduced in Australia and Europe differed in their passage histories and in the symptomatology produced in *Oryctolagus* rabbits, but both almost invariably produced lethal infections.

A single introduction of virus was made in Europe, whereas in Australia there are annual inoculation campaigns with the highly virulent virus, but in both continents the virus has established itself enzootically. Samples of virus from different parts of Australia and from Europe have been collected each year, inoculated into test rabbits, and grouped into one of five categories according to the mortality rates or the mean survival times seen in laboratory rabbits (Fenner, 1958b).

In spite of the annual reintroduction into Australia of highly virulent virus (causing a mortality rate of over 99 %) the majority of strains recovered from natural cases have been attenuated to a slight or high degree. Within a year of the first introduction of the virus, strains with a 90 % mortality rate had appeared. These have remained dominant ever since, but still more attenuated strains (causing mortalities sometimes as low as 20 %) have been recovered since 1955. In Europe the fully virulent strain has persisted longer and on a wider scale, but here, also, attenuated strains are becoming common.

When viruses are passed repeatedly in a particular host in the laboratory they often become more virulent for that host, although this may be accompanied by attenuation for some other (perhaps the natural) host. Attenuation for the passage host is virtually unknown. Yet in both Australia and Europe (and in many widely separated areas in each continent) the tendency has been toward a moderate degree of attenuation.

The explanation lies in the method of transfer of virus from one host to another. In the laboratory, the usual procedure is to select the animal showing the first signs of infection and to use material from this animal for the next passage; this procedure naturally tends to select variants with maximum virulence. In nature, however, the rabbit most likely to act as a source of infection for others is the one which offers large virus-rich skin lesions as feeding grounds for mosquitoes for the greatest length of time—that is, a rabbit infected by a strain of virus which does not terminate the infectivity of its host for others by killing it rapidly. This survival advantage of less virulent strains is presumably greatest when spread of the disease is least efficient—that is, during winter. In short, selection in the laboratory is for rapid multiplication and high final titer; in nature, it is for these properties and also for the property of persistence of the high final titer.

The results of titration of the superficial cells of the skin of rabbits infected with several strains of virus of different virulence showed that all naturally

occurring strains (whether highly virulent or not) multiplied to about the same extent and at the same rate. However, the highly virulent strains rapidly killed the host, whereas the common, moderately attenuated strains produced many skin lesions which remained infective for mosquitoes for a prolonged period.

A field experiment in Australia, in 1954, demonstrated the high survival advantage of the current strains (Fenner *et al.*, 1957). The highly virulent European strain of virus was introduced on a large scale at the beginning of the transmission season, in a rabbit population harboring the enzootic, attenuated strain. Although the virulent European strain dominated the peak of the epizootic, the local attenuated strain occurred in appreciable numbers at the end of the epizootic and was the only type of virus to survive through the ensuing winter.

There is, of course, a delicate balance between the genetic resistance of the host and the virulence of the virus. Tests on susceptible young wild rabbits, obtained each spring over a period of five years from rabbit populations exposed annually to severe epizootics of myxomatosis, showed that there had been a relatively rapid selection of genetically more resistant rabbits (Marshall and Fenner, 1958). It is interesting to speculate upon the effect of this change in host resistance on the selection of virus virulence: unless a variant appears which multiplies predominantly in the skin, causing very persistent virus-rich lesions, one might expect that more virulent strains will be selected in the future, as they may cause in partially resistant hosts the type of disease caused in susceptible hosts by the moderately attenuated strains of virus.

IV. Summary

In this article we have traced the factors determining virulence for the host from the simplest virus-host systems to the most complex and elaborate. Wherever possible, we have chosen examples to demonstrate the effect of each increase in order of complexity. But it is obvious that at the present time there can be no over-all synthesis. The type of problem considered in simple systems is quite different from that presented by complex systems, and it is impossible to analyze the virulence of viruses for complex systems in terms of those mechanisms demonstrated to operate in simple systems. This is particularly unfortunate, as most of the work on variation in virulence of animal viruses has, perforce, been conducted with the whole intact animal. All that can be done is to discuss the different levels of complexity offered by the various host systems which have been studied. We feel, however, that only in such terms will it be possible ultimately to create a science of animal virology.

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Chapter X

Serological Variation

T. Francis, Jr.

University of Michigan, Ann Arbor, Michigan

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I. Introduction

Actual demonstration of serological variation and immunological relationship among viruses has been frequently predictable from epidemiological observations of the behavior of a disease and its associated immunity. The protective effect of cowpox against smallpox, the recognition of alastrim, the development of vaccinia virus, and the knowledge of similar diseases in a variety of animals presented an array of circumstances inviting speculation and research into the nature of these variations. A groundwork was laid for consideration of a stem virus developing a variety of mutants under the influence of different hosts. Recurrent outbreaks of foot-and-mouth disease among the herds led to the first recognition of serologically distinct types in the first of the identified filterable viruses. Recovery from smallpox (variola major), measles, or yellow fever was, however, seen to be uniformly associated with prolonged immunity, indicating a high degree of serological unity in the respective viruses and also calling attention to the importance of

the pathogenesis of the viral infection upon the durability of immunity. The numerous recurrences of human influenza with repeated illness in the same individuals had led to accurate postulates of multiple strains in the suspected virus. Once serological variants of influenza virus were demonstrated, the gates were opened to the recognition of an interminable flow of antigenic alterations, naturally or artificially induced. In a reverse manner, expanding study of arthropod-borne animal viruses has brought together numerous strains of apparently different origin and nature into serologically related groups for which arthropods and lower vertebrates constitute major reservoirs. New families of viruses with numerous members and subgroups have been formed by virtue of common and distinct antigens, i.e., Coxsackie viruses, adenoviruses, and ECHO viruses. In these, again, clinical and epidemiological features of their behavior have served as pointers to their serological similarities and differences. Still other older viruses, such as poliomyelitis, have been divided into separate types with recognized epidemiological differences in behavior. Serological variation is now the rule among animal viruses rather than the exception, as was true twenty-five years ago.

Much of this development has been closely related to great improvement in techniques which provide high concentrations of virus without large masses of foreign protein, in the greater opportunity for quantitative studies, and in the greatly enlarged body of investigators concerned with the biological relationships among viruses which are causative agents of disease. Consequently, the disease manifestations with which the viruses are associated constitute a primary focus from which variations can be observed, while relationships among viruses from apparently divergent disease states are based on biochemical characteristics which determine their serological or cytopathological behavior. Conversely, the range of deviation from a common biochemical base will express the group boundaries. Thus, any aberration from the prototype represents a variant, but because of the unstandardized conditions under which differences are observed, their meaning is at times uncertain.

II Serological Reagents

Serological behavior of viruses, as with other antigens, is the resultant of a number of influences which participate in the reaction. It embraces the nature and source of the agent, the character and origin of the serum, and the method of measurement itself.

The virus preparation is ordinarily a collection of particles which may possess different degrees of reactivity. The size of the inoculum used can affect the amount of noninfective virus present that can combine with antibody, but is not reflected in titrations of infectivity. The nature of the

tissue in which virus is propagated undoubtedly affects the fullness of antigenic expression and contributes tissue components which may be antigenic in another species. If virus is inactivated before use in serological tests, the antigenic character may be altered.

Antibody response in the animal undergoing active infection can be expected to reflect the full array of viral components, especially if extensive tissue involvement occurs. Activities related to adaptation and virulence are not well recognized serologically as yet. Parenteral injection of nonmultiplying virus in animals may presumably anticipate a narrower serum response, but hyperimmunization consistently increases the breadth of reaction with secondarily related strains. Susceptible and nonsusceptible species may differ in this respect. The time of collection of serum from the test animal has been consistently shown to affect its reactive character; antibodies measured by complement fixation (CF) or hemagglutination inhibition (HI) may appear in workable amounts at different times from neutralizing, agglutinating, or precipating antibody. This is particularly true after infection. The antibodies may not have the same immunological significance if they are reacting with constituents of different functional and biochemical character. Thus, the technical procedures must be conducted with awareness of the nonspecific as well as specific reactants of serum, the character of the virus preparation, and the quantitative considerations which permit valid interpretations.

Since influenza viruses have been most extensively studied and illustrate the numerous features involved in the interpretation of serological variation and its significance, the ensuing discussion will be largely limited to that group of viral agents.

III. Serological Variation Among Influenza Viruses

A. Viral Structure

Serological behavior of influenza viruses has led from the early periods of antigenic study to the suggestion that characteristic antigenic components of a strain were located at or near the surface of the virus particle. Examination of the chemical structure of influenza A virus has been particularly pursued by Hoyle and his co-workers (Hoyle, 1952; Hoyle and Frisch-Niggemeyer, 1955; Frisch-Niggemeyer and Hoyle, 1956). The virus particle is considered to consist of a central core of type-specific nucleoprotein (RNA), or "soluble" antigen, surrounded by strain-specific mucoprotein (hemagglutinating, immunizing antigen) bound together by lipo- and mucoprotein, presumably derived from the cell wall. The serological behavior of this matrix is essentially unstudied, but one may speculate that it plays a role in certain of the phase variations by covering active surface antigens, and may also be involved in nonspecific reactions.

The complement-fixing antigen common to the type A strains (type-specific) differs from that of type B and may be used for typing of strains when immune serum to the test strain is not available. It may be found in fluids as "soluble" antigen. Strain-specific complement-fixing antigen is probably the same as the hemagglutinin of the virus particle. Some investigators have found it sufficiently distinctive as to parallel the specificity of hemagglutination or neutralization. The hemagglutinating mucoprotein combines specifically with antibody and appears to be an essential participant in the infectious process. The character of primary and secondary antigens, their number and composition would then be considered as variations within the molecular configuration of these hemagglutinating units. The host and his immune state may exert a significant influence upon their make-up, hence upon the character of the virus strain. The RNA is considered to be the basic genetic replicating component. Its role in infection and immunity is uncertain. The presence in virus preparations of components derived from the host tissue introduced antigens which can give common but extraneous serological reactions, whether they are integrated into the virus or are merely contaminants.

B. Serological Types

Three immunologically distinct types of influenza virus, A, B, and C, have been generally acknowledged because of their association with human disease of related clinical nature, their comparative behavior in other hosts, and the similarity of their action in hemagglutination. It has been proposed for similar reasons that the Sendai virus or hemagglutinating virus of Japan (HVJ) (Kuroya *et al.*, 1953) be considered type D (Jensen *et al.*, 1955; Francis, 1955). This has been supported by Russian investigators, who observed an epidemic of influenza in 1956 in which this virus was involved (Gerngross, 1957; Gorbunova *et al.*, 1957). More recently Chanock and others (1958) have reported isolation of the agent from children in the United States. In addition, they have encountered another agent in outbreaks of respiratory illness resembling influenza, which they tentatively suggest may be a type E.

C. Antigenic Analysis of Type A Strains

1. *Studies with Sera of Experimental Animals*

a. Initial Studies in Mice (1935–38). Influenza A has been the most frequently epidemic since 1933, when the virus was discovered by Smith and associates (1933). Strains of the same virus were then isolated in North America (Francis, 1934) and in Australia (Burnet, 1935). The swine influenza virus (Shope, 1931) was readily recognized by active immunity tests to be related, but different serologically. Its relationship to strains from man was

further established by the demonstration that the serum of ferrets or mice given repeated inoculations of the PR8 strain developed neutralizing antibodies to the swine virus, and vice versa (Francis and Magill, 1935; Francis and Shope, 1936). It was also noted that the serum of some persons contained neutralizing antibodies to swine influenza virus, indicating further a relationship between the viruses of the human and swine influenza (Andrewes *et al.*, 1935; Shope, 1936), while convalescent human serum reacted equally well with both viruses in the complement fixation test (Smith, 1936; Hoyle and Fairbrother, 1937).

At this time it was demonstrated that serological differences existed between strains of human origin (Magill and Francis, 1936). The epidemic of 1936–1937 provided a number of additional strains, and a series of studies towards antigenic analysis ensued (Magill and Francis, 1938; Francis and Magill, 1938; Smith and Andrewes, 1938; Burnet, 1937). It became apparent that a number of antigenic components existed in varied patterns and with extensive overlapping among the available strains. Under the conditions of study, however, it was noted that some strains presented greater serological individuality than others; some were poor antigens; some reacted as well or better with serum against heterologous strains than with homologous serum. It was clearly demonstrated that antiserum taken from the test animal early after inoculation had a high degree of strain specificity, while a later specimen or hyperimmune serum demonstrated by its broader effect the multiple relationships between the various strains. The results emphasized the importance of reciprocal cross testing, since the serum against one strain might neutralize the homologous and a given heterologous strain, but serum to the latter would have little effect upon the first, so that different impressions would be gained if only one of the sera was used. Although some suggestions were offered for grouping these strains as types (Burnet, 1937; Smith and Andrewes, 1938), the limited knowledge scarcely seemed to warrant more than rough groupings, since the characteristics of one group tended to merge into those of another. In general, the strains from one epidemic period in different parts of the world closely resembled one another; those from 1935–36 seemed to differ somewhat from those of 1936–37, but exhibited more relationship with the PR8 strain of 1934 than with the original WS strain of 1933. One of the English 1936–37 strains (Gatenby) resembled the 1935 strains. Two other strains closely resembled WS. The relation of swine influenza virus to the human strains was more distant. The observations made by neutralization tests in mice agreed quite well with those from reciprocal cross immunity tests in vaccinated mice with the various strains, although the latter procedure tended to bring out a broader group immunity. It was suggested by Magill and Francis (1938) that the virus particle of a strain might well have a surface composed largely of the dominant antigen,

while the interior or more inaccessible portions contained other antigens, and yet, because of the variation in amount or arrangement, each strain or group of strains could be serologically different. Strains at each end of the spectrum might thus exhibit closer relation to a third mid-strain than to each other. Because the studies were done exclusively with virus that had been adapted to mice, some doubt still remained as to whether they reflected the state of native human strains, although no differences were detected between early and late passages of mouse or tissue culture lines (Francis and Magill, 1938).

b. Analysis by Hemagglutination-Inhibition. In succeeding years a number of investigators, using sera of various kinds, analyzed the serological character of strains isolated from man over various segments in time. The majority used hemagglutination-inhibition techniques (Hirst, 1942) and virus cultivated in embryonate eggs. Hirst (1943) employed strains isolated directly in eggs with convalescent ferret sera. Efforts were made to correct for the variation in sera obtained from different animals, in different lots of erythrocytes, and in the combining power (avidity) of different strains of virus. Studying 18 strains derived from the 1940–41 epidemic, he pointed out the extraordinary homogeneity of the group with no difference between those isolated at the beginning or end of the outbreak. The strains clearly diverged, however, from the earlier WS, PR8, or even 1936–37 strains. Study of strains from the 1943 epidemic showed only small differences among them or between them and the 1940–41 strains (Hirst, 1947), indicating a great antigenic stability of epidemic strains.

In 1947, strains recovered from epidemic influenza A in the Northern Hemisphere were promptly recognized by a number of investigators to represent a new variant, and it was learned that a strain (Cam) isolated in Australia during the preceding winter was of the same character. These strains extended as the predominant influenza A infection throughout the world. The 1947 strains were related to earlier strains by common components, but the dominant group antigen differed more distinctly than had been the case among strains of intervening years. Immune serum from any source demonstrated the individuality and limited cross reaction with other strains. Furthermore, vaccination of man with PR8 1934 and Weiss 1943 strains stimulated no antibody response to the 1947 strains (Francis *et al.*, 1947), but vaccination with a prototype 1947 strain FM1 did induce increased antibody production against PR8, Weiss, and later strains (Francis, 1952; Meiklejohn *et al.*, 1952). The one-way serological cross reaction was also exhibited with sera of experimental animals. This group of strains was designated A-prime by the Commission on Influenza (1948; Salk and Suriano, 1949).

In the next several years more extensive serological analyses of type A strains were reported. Van der Veen and Mulder (1950) measured ferret sera

against 39 type A strains from 1933 to 1949, and swine influenza virus against prototype strains of 1933, 1934, 1941, and 1947. The swine virus and WS strain were classified as distinct variants. The authors grouped strains from 1934 to 1945 together because of extensive cross reactions, while differing from later and earlier strains. But they comment on the great individuality and the difficulty of classification within the group except by cross testing with sera of significant heterologous potency. The 1947 and 1949 strains differed from the PR8 group but were closely similar to each other. The same relation between 1949 and 1947 strains was noted by Chu *et al.*, (1950). Hilleman (1951, 1952) introduced an orderly effort to chart antigenic make-up by quantitating HI activity of immune chicken serum to a strain against each of four prototypes—1933a, 1934b, 1947c, 1950d. The patterns were expressed as shown in Table I.

TABLE I

Strain	Year	Type	Configuration
WS	1933	A	$a_3\ b_1\ c_0\ d_0$
PR8	1934	A	$a_2\ b_3\ c_0\ d_1$
FM1	1947	A	$a_1\ b_0\ c_3\ d_3$
Hume	1947	A	$a_0\ b_2\ c_3\ d_0$
Most strains	1949–51	A	$a_0\ b_0\ c_1\ d_3$

Thus, the basis for cross reaction can be visualized. Again the differences between groups were observed, and the great homogeneity of strains from different parts of the world during a twelve-month period in 1950–51 was noted. But, in addition, attention was called to a series of French, Canadian, and Arctic strains of 1949 resembling PR8; there were other suggestions that strains of earlier configuration might be in circulation in later years. Magill and Jotz (1952) studied with rabbit sera a large number of strains isolated over the same period. The results were similar to those of Hilleman, and on the basis of variations in reaction with selected strains, the strains of 1946–47 to 1949–50 were grouped together, while essentially all the 1950–51 strains were congregated in another. Neither series contained significant representation of 1940–41 and 1943 strains. Five subgroups of type A virus were suggested. Isaacs and Andrewes (1951), using ferret sera for study of 1950–51 strains, reported two subgroups which differed in avidity for immune serum and were thought to be epidemiologically independent. It is doubtful that the reported differences are of antigenic nature, since they are observed only with ferret sera. Serologically, they were not observed by Hilleman or Magill, who considered their 1950–51 strains serologically homogeneous. A number of investigators have described a further antigenic deviation in 1953 strains, suggesting that they were a new group unrelated to 1947 and 1950 strains

(Mulder *et al.*, 1956), although their results continue to show cross relationships at low levels. Takatsy and Hamar (1955) insist that the 1953 strains are direct mutational descendants of a 1952 strain.

c. Analysis by Antibody-Absorption. Friedewald (1944) first employed antibody-absorption methods effectively in antigenic analysis of 6 influenza strains. Concentrated virus from allantoic fluid was used to absorb convalescent ferret and convalescent or postvaccination human serum. The effect on homologous and heterologous antibody was measured in cross tests by mouse neutralization, HI, and CF methods. Each strain removed all antibody from its homologous serum and varying amounts of cross-reacting antibody from the different heterologous sera. Clear differences and relationships between the test strains were demonstrable. It is of interest that the PR8 strain removed antibody against all strains from convalescent or vaccinated human serum at that time, while other strains exhibited more strain-specific effect. The patient's own strain also removed all antibody from his convalescent serum although it differed somewhat from PR8. Antibody as determined by all three serological methods was equally affected.

Hirst (1952) reported antigenic grouping by antibody absorption of 61 strains covering the 18 years from 1933–51. He employed hyperimmune rabbit serum against swine 1931, WS 1933, PR8 1934, Melbourne 1935, Talmey 1937, Alabama 1941, and FM_1 1947 strains. Each serum was absorbed with heterologous strains until only antibody to the homologous prototype virus remained. Each strain was tested by HI against the 7 strain-specific sera. This represented, therefore, largely a one-way grouping. Swine, WS, and PR8 groups appeared distinct, while strains from 1935–37 were divided among Melbourne, Talmey, and Alabama 41 groups. The latter also included all strains from 1939 to 1944; the strains from 1946 to 1950 were in the FM_1 group. The strains of 1950–51 were separate. Certain strains from different periods were not classifiable.

Antigenic analysis was extended by reciprocal serological reactions with sera absorbed through periodate-treated virus fixed to formalin-treated cells (Jensen and Francis, 1953). The absorptive capacity of each cell-virus complex was standardized against homologous immune serum and equal absorptive amounts were then used for comparative observations. Sera of ferrets convalescent from infection with one of 18 strains were pooled in quantities which provided a pool with equivalent HI titers against each strain. Absorption with a given strain removed antibody to itself, whether the antibody derived from homologous serum or from cross-reacting antibody from sera against other strains. It reduced the titers to heterologous strains presumably proportionate to the amount of the same antigens shared by it and heterologous strains represented in the antibody pool. Thus, a profile of the quantitative and qualitative antigenic composition of a strain was obtained. Within

the 29 strains studied, 18 different antigens were detectable, and as many as 15 could be demonstrated in a given strain. Takatsy and Furesz (1954) have raised some technical objections to the procedures from which these conclusions were drawn. They accept, however, the principle of common antigens. The strains tended to group chronologically according to major antigens. The older strains exhibited limited crossing with strains of later years, but the latter frequently exhibited numerous common components with strains of the earlier years. It is clear that an adequate view of antigenic composition and relationships is obtained only by testing each virus against numerous sera reflecting the composite antigenic make-up of the individual strains. The variation in strains from different epidemic years or periods can thus be seen in relation to the antigenic spectrum as a whole. When strains from different periods were compared by the same procedure, it was noted that, despite individual variations, strains from 1933 to 1943 exhibited distinct similarities and that the deviations among the group were chronologically random rather than showing a directed mutational shift in the accession of new antigens with succeeding years. The same situation obtained with a representative series of strains isolated in the years 1946 to 1955. Strains of swine influenza virus over a 23-year period were, with two exceptions, essentially identical (Jensen and Peterson, 1957).

d. Interpretation. The substance of these analyses is to emphasize the presence of numerous antigenic constituents common to a large proportion of strains of influenza virus type A; the dominant antigen of one may be so much reduced in another as to be detectable only by procedures which disclose the full antigenic complement. While one component becomes enhanced as a characteristic strain antigen, others may recede to represent only secondary or group antigens. Variation in strains is viewed then as primarily a process of quantitative or spatial rearrangement, rather than complete loss of existing antigens and the development of completely new antigenic structures. Rearrangement of 18 antigens of itself permits an almost inexhaustible supply of variants. The bulk of evidence is in support of this thesis, but some investigators prefer the loss and gain motif (Andrewes, 1954, 1957).

The appearance of the 1957 Asian variant brings another abrupt transition which, on the basis of present information, suggests a sharp mutational shift, bearing little similarity to recent A-prime strains or to isolated strains from earlier years. Studies of its antigenic character are not sufficiently advanced to portray its make-up, but observations with human serum demonstrate that it is related to strains of earlier distribution.

Hilleman (1954) has pointed out that in these efforts toward analysis and classification the procedures employed and the materials used have differed so widely as to make interchange of the data difficult. Certain generalizations

appear, nevertheless. Each strain may exhibit individual characteristics, but those from an epidemic season have great similarity. For a period of years after the appearance of a major antigenic variant, there is a tendency to divergence in the strains of ensuing epidemics. This appears to be a random variation within the group rather than an orderly progression. The variations of themselves are of general biological and immunological interest, but their epidemiological significance must clearly be gained from study of the disease in man.

2. *Studies with Human Sera*

a. Response to Infection. After the demonstration of the development of specific neutralizing antibody to influenza virus in convalescent patients (Francis and Magill, 1935), attention was directed to the specificity of the response to infection with known strains and also to the antibody content of serum from the general population. Andrewes and associates (1935) observed that antibodies to swine influenza virus were present in the serum of 100 % of a group of human subjects over 15 years of age, but in none under 10 years, whereas antibody to the human WS strain was found in both children and adults. Similar results were obtained with the PR8 and swine strains in serum from the American population, and with WS, Melbourne, and swine strains in Australian subjects (Francis and Magill, 1936; Shope, 1936; Burnet and Lush, 1938). The suggestion was made that the swine antibodies resulted from infection with virus of the 1918 pandemic, which had then become established in swine (Laidlaw, 1935; Shope, 1936). On the other hand, it had been well established that repeated exposures of experimental animals to human strains gave rise to antibodies to swine virus. In St. Helena, where the 1918 epidemic was said not to have occurred, sera of adults who had never left the island contained, in 1935, little antibody to swine virus. When an epidemic of a WS-like influenza infection occurred in 1936, antibodies to WS strain promptly rose and, in addition, swine antibodies also appeared, even in persons who showed none previously (Stuart-Harris *et al.*, 1938). This seemed to indicate a response induced by common antigens in adults with broad serological reactivity. Rickard and associates (1945) noted, moreover, the development of swine antibodies in infants recovering from presumably their first infection with influenza A, although Hare and Riehm (1941) had pointed out that it was more likely after the first decade of life.

As knowledge of strain variation grew, there was increased attention to the use of multiple strains in the measurement of antibody response to infection and vaccination. It was recognized that the response of children was more strain-specific than that of adults. In 1947, however, vaccination of adults with the PR8 and Weiss strains enhanced titers to those strains but not to the oncoming A-prime strains, but patients recovering from infection with

A-prime strains developed good titers to both A-prime and PR8 strains (Francis *et al.*, 1947). However, a significant proportion of children vaccinated with PR8 virus in 1948 developed antibodies to A-prime strains or even to swine virus (Quilligan *et al.*, 1948). Thus, while serological studies with animal sera were describing strain changes in different years, the studies with human sera continued to emphasize the lack of strain specificity, especially in the response to infection (Horsfall and Rickard, 1941; Anderson, 1947; Magill and Sugg, 1944). Nevertheless, the initial and convalescent titers were frequently found to be much lower when tested with strains from a current epidemic than with older stock strains. This is illustrated in data from patients in 1947 (Table II).

TABLE II

Group	No.	PR8 titer		Rhodes 1947 titer	
		Acute	Conv.	Acute	Conv.
Unvaccinated	39	76	230	27	120
Vaccinated PR8 and Weiss	55	499	809	32	115

b. Age Distribution of Antibody to Different Strains. That the major antigens representative of the A-prime strains were not new to the population was shown in the fact that gamma globulin prepared in 1943 and 1944 had demonstrable antibodies to strains isolated in the years from 1947 to 1951; material prepared after 1947 showed fourfold increases in titer to these strains and to the PR8 strain, while the already high titers to WS and swine strains were unchanged (Davenport *et al.*, 1953).

A systematic examination of antibody to strains of different years throughout the age span of the general population in 1952 revealed three distinctive patterns (Davenport *et al.*, 1953; Francis *et al.*, 1953). The sera of children contained antibodies essentially oriented to the A-prime strains, which had been and were currently prevalent. This had been noted also by van der Veen (1951) and Hilleman (1954). They were highest in the five to twelve year old groups, but after the age of seventeen to eighteen the titers to these strains were low. Antibody to the strains of 1933 to 1943 appeared about the twelfth year of age, but was at its highest between seventeen and twenty years, and then leveled off for the older ages. Antibody to swine influenza was first seen at twenty-nine years, reached its peak in the age group thirty-five to thirty-eight and then settled at a lower level. The first two patterns correspond in time with established prevalence of known serological groups of type A virus and hence permit the inference that virus antigenically similar to swine

influenza virus was the predominant strain during the period of 1915–24, especially about 1918. In 1935, antibodies to PR8 were found in the first years of life; in 1948, they were found first at the age of seven; in the 1952 sera they were not measurable until eleven or twelve years of age. In 1935, antibodies to swine virus were not found before eleven years of age; in 1952, seventeen years later, they were first detected at twenty-nine years of age. The shift of these antibodies temporally coincides remarkably with the passage of time, and the age distribution of antibodies to the respective prototypes thus constitutes a recapitulation of the periods of prevalence of certain antigenic groups of type A virus. The same patterns have been obtained in England, Japan, and Czechoslovakia (Davenport *et al.*, 1955; Davenport and Hennessy, 1958; Blaskovic and Rathova, 1956).

Recognition of these antibody patterns in the general population provides epidemiological perspective to consideration of serological classification and appears to define the significant variants among influenza A viruses. They behave as major serological groups in which the lesser degrees of serological variation from one year to another are indistinct. The data also afford explanation for the variability and lack of strain specificity in antibody response to infection or vaccination; existing antibodies to one group are enhanced by subsequent experience with a related strain. High antibody levels noted in the acute stage of infection are more likely then to be the heterologous pre-existing antibody to one group, while that to the infecting virus is low.

c. Doctrine of Original Antigenic Sin. The dominant character of the group antibodies is further emphasized by serological responses of different age groups to vaccination. When children who have some antibody are vaccinated with any of the A strains, they respond with antibodies to A-prime virus strains; persons in the middle range respond with additional antibodies to the PR8 group of viruses; people over thirty respond with increased antibody to swine influenza virus (Davenport and Hennessy, 1956). Absorptions of sera from groups of persons both normal and after vaccination resulted in complete removal of antibody to all strains of influenza virus within a type when a strain of antigenic configuration similar to that of the strain of first experience was employed (Jensen *et al.*, 1956). Absorption of sera from people of twenty years of age with the PR8 strain removes antibody to all other type A strains; all antibody in the serum of persons of thirty to fifty years is absorbed by swine influenza virus; all antibody is absorbed from children's serum by A-prime strains. These effects were substantiated by observations in ferrets successively infected with three serologically different strains of type A virus. Absorption of the serum after the third infection with the second or third strain removed antibody to that strain and to some cross-related antigens, but when the serum was absorbed with the first infecting strain, antibody to

all three strains was removed. The first experience with a type A influenza virus conditions the antibody-forming mechanisms so that the related antigens in strains of all subsequent experiences stimulate antibody of the initial character, which then represents the age group throughout its life. With increasing age, characteristic strain antigen is less apparent in the antibody response to infection or vaccination because antibody to most of the antigens is already present and a broader response takes place. This apparently provides a resistance which is reflected in the reduced age incidence in older persons to all epidemics of influenza A. One important observation is that conditioning to antigens not represented in the antibody content of an age group can be established by vaccination with appropriate strains (Davenport and Hennessy, 1956). Subsequent inoculations or exposure will result in enhancement of that antibody as well as to the first antibody acquired naturally. Antibody patterns can thus be developed in all age segments of the population to provide a broad coverage against numerous antigens and strains. These observations have led to formulation of the doctrine of "original antigenic sin" (Francis, 1955; Davenport and Hennessy, 1956).

The 1957 Asian strains of type A differ distinctly from those of the last twenty-five years, and antibodies to them are not found in most of the population. However, as originally observed by Mulder (1957; Mulder and Masurel, 1958) and repeatedly confirmed, persons of seventy to eighty years of age may have antibodies to them. This indicates that the Asian strain had been in circulation some seventy years ago, which would approximate the time of the 1889–90 epidemic. Moreover, persons above forty especially above seventy, respond to vaccination with Asian strains by better antibody production to that dominant antigen than do younger age groups. Some produce antibody to Asian virus after vaccination with heterologous strains (Davenport, 1958). The incidence of disease has been distinctly lower in the population over forty years. Antibody to the 1957 strains apparently typifies the oldest age group and will hereafter be typical as well of that population cohort which is now in the early years of life.

3. *Classification of Type A Strains*

The accumulated information from serological studies of influenza A virus provides a reasonable basis for separation of the strains into four groups, each representing a period of epidemic prevalence. They constitute major mutations, but in each instance their dominant antigens have been shown to be related to strains dispersed in other years. Genetic controls appear to keep them within the type boundaries rather than following an unrestricted course of developing totally new species. (Table III).

Subgroups showing less distinct variation from each other occur within the major groups and represent random deviations about initial prototype strains of the group (Jensen and Peterson, 1957). This variation appears more as an immunological drift (Burnet, 1953) in the relative expression of their multiple common antigens rather than a directional shift.

TABLE III

INFLUENZA VIRUS, TYPE A

Group	Prototype	Common term	Prevalence
A_1	S15	Swine influenza	19— to 1928
A_2	PR8	Influenza A virus	1934–43
A_3	FM1	A-prime virus	1946–57
A_4	Japan 305	Asian influenza	1957 (188?–189?)

The differences between groups A_2, A_3, and A_4 and the similarity of strains within the groups is further illustrated by the effects of vaccination. Vaccine prepared from one of several strains may protect against epidemics related to strains of different subgroups, but strains from one group have not been highly effective in protection against disease caused by strains of another. Nevertheless, evidence indicates that polyvalent vaccine of certain groups can provide a significant degree of protection against strains of a heterologous group (Commission on Influenza, 1957). And it seems likely that the essential antigens representative of all type A strains may be accumulated into one vaccine which will provide a composite immunity to the entire type.

The extensive strain relationships and the limited number of major variants appear in keeping with the hypothesis that a finite number of antigens is represented in type A virus and that variation is primarily rearrangement of the constituents. Moreover, if antibodies and immunity of the population are significant factors in the determination of the variants which arise, recurrent or cyclic resurgences of an antigen can be expected. The young segment of the population is always the most susceptible and it has been increasingly deficient in antibodies to the swine group, the PR8 group, and, until recently, to the Asian group of strains. Immune pressure might then direct variation into this wide gap where numerous alterations could be effectively distributed. The Asian variant of 1957 seems to have been so derived. There remains, however, the possibility that the major variants are recurrences of fixed strains which have remained antigenically intact in a reservoir or by scanty distribution, then to regain epidemic status when opportunity permits. The return of virulence would be more an influence of the host population than of antigenic variation as currently recognized. The

fact that antibody to swine strains is sporadically detected in the serum of young children might indicate that a strain of that group is still circulating in the human population. The 1949 epidemics in the Arctic and elsewhere, from which PR8-like strains were reported, could be considered a lagging virus, although the possibility of laboratory contamination has not been excluded. If long-term maintenance of antigenically unchanged variant strains is established, as may be the case with swine influenza, the concept of continuous variation as a basis for recurrences would obviously need reconsideration.

D. Variation in Type B Influenza Virus

Variation in type B virus has occurred in a manner resembling that of type A. Suggestions have been repeatedly made that observed differences warrant group divisions. Some differences in age distribution of antibodies to different strains have been observed (Davenport *et al.*, 1953). Interestingly, the 1952 strains appeared somewhat more closely related to the LEE 1940 strain than did the 1945 strains. Vaccination in 1945 with LEE strain provided excellent protection (Francis, 1950), and vaccination of children with LEE strain in 1952 protected against influenza B of that year (Hennessy *et al.*, 1953). In 1955, vaccination with LEE induced poor antibody to the 1954 and 1955 strains and immunity to disease was of low order (Davenport and others, 1956). It is suggested, therefore, that group B_2 begin with the 1954 strains (Table IV). There is little information regarding differences among strains of other types.

TABLE IV

Group	Prototype	Prevalence
B_1	Lee	1936–48
B_2	GL	1954—

E. Serological Variation during Passage

1. *Adaptation to New Hosts*

Because the studies of 1936 were made mostly with strains that had been isolated and maintained through animal passage, a suspicion persisted and became a general belief that a number of the serological alterations that were observed were modifications resulting from animal passage. Hirst (1947) noted in the adaptation of two strains of virus to mice and eggs that the mouse line of one of them developed a distinct serological deviation, as compared with the egg line. The mouse line also appeared to differ from that of the second

strain, which was originally closely related. Little alteration of the second strain was noted with adaptation to the mouse lung. Sugg (1949) also noted distinct serological variation in the Cam strain adapted to mice; this difference was more that of an increased antigenic and antibody combining potency of the mouse line than any evidence of a major antigenic deviation. A second passage series carried out with the same strain became pathogenic for the mouse lung without any evidence of serological alteration. No antigenic variation was observed with certain other strains systematically studied in the process of adaptation to mice (Wang, 1948; Davenport and Francis, 1951). On the other hand, in 1947 a serological variant of the PR8 mouse line was detected in virus which had been transferred to and maintained in tissue culture. The variant was still clearly PR8 in character but differed serologically and was sufficiently distinct so that mutual cross-protection did not occur in vaccinated mice. Extended studies have shown, however, that serological differences are clearly detectable between strains newly isolated in eggs or other hosts from the same outbreak, so that the antigenic variations observed in the earlier antigenic studies cannot be attributed primarily to animal adaptation but seem properly to represent inherent differences in the strains themselves. In 1952 Hirst considered the modification he noted earlier during adaptation to mice to be of little moment. In fact, the mouse lines often appear to display the proper antigenic structure of a strain more completely. Mulder *et al.* (1956) have pointed to the fact that numerous antigens may be hidden in egg passages which show up in the mouse lines. The behavior of the same initial strain of virus in a variety of hosts was studied by Jensen *et al.* (1957). Lines of virus from a single human gargling were initiated and maintained in eggs, ferrets, mice, hamsters, and tissue cultures of allantoic membrane. Alterations were looked for at intervals in the course of adaptation by using the different lines as antigens for production of antiserum in the homologous and other species of animals. A variety of variations was observed but the most prominent anomalies were in the tissue culture and mouse or hamster lines. The tissue culture line was the most line-specific serologically, combined in hemagglutination-inhibition tests least readily with antibody even to homologous serum, was the poorest antigen, and exhibited the least ability to absorb antibody from pooled serum. The mouse and hamster lines were the most efficient in these respects. The possible antigenic variations which were observed were unstable and passage of the tissue culture line in mice or of the mouse line in eggs resulted in restoration to the original basic pattern. Review of the results strongly suggests that the differences are related to the nutritional adequacy of the medium in which the line was propagated. The behavior of the tissue culture and egg strains may well be expression of a starved or poorly developed virus, while the mouse and hamster lines appear well fed with much more complete

expression of the total antigenic complement. The differences seem to be more quantitative than qualitative alterations in composition.

2. *Induced Variation*

a. In Eggs. Several studies have reported the alteration in antigenic behavior of strains grown in an environment of antibody (Archetti and Horsfall, 1950; Isaacs *et al.*, 1952; Burnet and Lind, 1954; Edney, 1957). Horsfall (1952) has especially commented that in their study the results were obtained when large amounts of virus were introduced, just as is true of production of "incomplete" virus. On the other hand, Magill and Jotz (1952) have recalled the unsuccessful efforts made earlier to induce such changes with immune serum in tissue culture. A number of the variations have apparently stabilized so as to persist in the absence of immune serum. Nevertheless, they are of limited character and, as suggested above, may be quantitative rather than truly constitutional.

b. In Mice. Gerber and associates (1955, 1956) detected four successive generations of antigenic variants of PR8 strain, each derived from the previous one by serial passage in the lungs of mice immunized with the homologous agent. The variants exhibited progressively decreasing reactivity with the parent PR8 antiserum, while retaining the ability to elicit antibody to the original PR8 strain and to their respective predecessors. These were stable mutants. Antibody-absorption tests revealed that they differed from the original in the appearance of previously unrecognized antigenic components. Nevertheless, vaccination of mice with PR8 strains protected against the variants. Magill (1955) also reported the recognition of variants developing in immune mice; the nature of these he considered to be antigenic rearrangement. An important feature of these observations again is to emphasize that the shifts occur within limited boundaries of related structures in association with immunological pressures.

3. *Phase Variation*

One of the difficulties in evaluation of serological variations which can be demonstrated is the presence of other substances in serum which can interfere with serological activity of the virus and simulate antibody. One of these associated with mucopolysaccharides of serum has been extensively studied (Gottschalk, 1954, 1957). It was shown (Francis, 1947) that if influenza virus is heated, its capacity to combine with erythrocytes is inhibited to high titer by normal serum. Some strains, especially when first isolated in eggs, are especially susceptible to normal serum inhibitors so that their antigenic pattern is difficult to determine promptly. This exaggerated tendency to combine with inhibitor may be a reflection of epidemic capacity, indicating a high ability to combine with receptors of any kind. This effect may be

increased by passage in certain tissues (Jensen *et al.*, 1957), while passage in mice may decrease susceptibility to the inhibitors. Another heat-labile effect of serum which neutralizes virus infectivity was observed by Ginsberg and Horsfall (1949) in mice. This has been studied in detail by Chu (1951) and Tyrrell (1954). The active agency appears to belong to the class of natural antibodies and is found in the α-globulins. Here again strains and lines may vary considerably in susceptibility. Adaptation of strains to mice is associated with development of resistance to this inhibitor, but the Asian 1957 variant was initially refractory.

Early observations with neutralization or active immunity tests showed that strains varied in their reactivity with homologous and heterologous sera (Magill and Francis, 1938; Francis and Magill, 1938). Hirst used the term "avidity" for differences in antibody combining power among strains and pointed to its influence upon serological definition of strains by hemagglutination technique. After systematic study, van der Veen and Mulder (1950) classified strains in P, Q, and R phases according to their behavior in hemagglutination-inhibition tests with homologous and heterologous ferret sera. P phase virus is inhibited to high titer by its homologous serum only. Q phase virus is poorly neutralized even by the homologous serum, while the R phase is inhibited to high titer by homologous sera and by sera against heterologous but related strains. They may be altered by passage in mice from Q to P phase. Isaacs *et al.* (1952) reported change from P to Q phase when immune serum was introduced into egg passage, but have also reported that newly isolated strains may breed true in egg passage. The observations of Jensen *et al.* (1957) have pointed out, however, that the nature of the host tissue may be a distinct influence and phase alterations may in reality represent quantitative variations. Isaacs (1953), however, proposed that the P-Q difference might reflect changes in steric relationship of antigens, while the observations of Fiset and Depoux (1954) with cross antibody absorption led them to suggest that the differences are related to the presence of masked antigens unavailable for antibody union and that these may be unmasked selectively when passed through an immune host. This same unmasking may conceivably result also from passage in an adequate animal host. The concept is in keeping with the idea of antigenic ebb and flow within the virus particles. The ready reversibility of the changes under a variety of conditions invites speculation that they relate to quantity of surface lipids which binds the specific antigens into a functional unit; Mulder *et al.* (1956) have speculated that they may reflect some intervening biophysical property separate from antigenic structure. Since these effects are primarily seen with ferret antisera, not with chicken and mouse sera, the influence of such factors on serological studies of antigenic classification may result in attention concentrated on what are antigenic artifacts in mouse or egg lines rather than on true views of

antigenic constitution. The latter refer as R-S relation to the situation in which two strains may be identical in one direction but differ markedly in another. It is seen between mouse and egg lines of the same strain, lines of the same strain from different laboratories, and in strains from the same epidemic. It emphasizes the need for multiple cross reactions to obtain a reasonable view of strain relationships, particularly when mouse lines may disclose hidden antigens in the egg lines.

Another phase of serological variation under active study at present relates to antigenic and genetic recombination among strains. It is discussed elsewhere in this volume.

IV. Variation in Arthropod-Borne Viruses

A. Demonstration of Strain Relationships

Knowledge of these "arborviruses" has advanced progressively, with the result that instead of a diffuse array of individual items of diversified origin, they are congregating as groups of antigenically interrelated strains. The differences may well be imprints made on a basic antigenic configuration by the respective hosts and vectors in which they are maintained, just as individual human hosts influence the strains of influenza virus they entertain. This recalls Hammon's (1948) description of a grandfather or stem virus. Although numerous investigators have contributed to the advance, the extended observations of Smithburn (1952, 1954) with the neutralization test in mice first demonstrated a series of no less than 12 interrelated agents whose cross-reactions were usually nonreciprocal; each of them had, however, an antigen in common with at least one other member of the group. The development of hemagglutinating techniques for the encephalitis viruses by Sabin and Buescher (1950), Chanock and Sabin (1953a,b,c, 1954a,b), Sweet and Sabin (1954), led to systematic study of relationships by Casals and Brown (1954), who formulated two sharply defined groups, A and B. Later Casals (1957) classified 47 strains of arborviruses with the addition of a group C of eight members, while eight others were as yet unclassified.

B. Value of Successive Exposure in Demonstrating Relationships in Virus Groups

Smithburn (1954) demonstrated that an animal immune to one virus of a group may respond to a second virus, not only by producing antibody against the second, but by producing more antibody to the first and also antibody capable of reacting with still other viruses. Diverse cross-reactions were noted with serum of human subjects convalescent from infection with one or

another of the viruses. These events have been well confirmed with serum of experimental animals, especially by Price (1956) and by Casals (1957) with hyperimmune mouse sera and hemagglutination-inhibition tests. They correspond to the results observed with groups of influenza virus, leading to the doctrine of "original antigenic sin." A specific response to infection is obtained in an inexperienced host but after previous infection by a member of the group, reinfection elicits hemagglutination-inhibition, complement-fixing, neutralizing antibodies and active immunity of a marked group character. Furthermore, it was observed here, too, that serum from an animal late after inoculation exhibited broader group coverage.

It can be emphasized that this basis for demonstrating mutual serological relationships is becoming generally applicable to viruses which may appear to be independent multiple variants, as measured by initial serological response. It has not as yet been explored in the complex system of adenoviruses, Coxsackie, and ECHO viruses, but it seems a fruitful area for study. Skinner (1953) has reported that infection with a second type of cattle immune to one type of foot-and-mouth disease virus results in antibody to the second type, but also sharply enhances antibody to the initial type. Vaccination of human subjects having antibodies to type 2 poliovirus alone with polyvalent poliomyelitis vaccine results in greater antibody rises to types 1 and 3 than occurs in persons without antecedent type 2 antibody; this illustrates a group response (Francis, *et al.*, 1957).

The results demonstrate that serological variation at apparently high levels of difference within the species still maintains a close relationship with other members of the species. This does not appear to be the result of a single common antigen, but of multiple shared antigens which contribute to serological specificity and commonalty.

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Chapter XI

Genetic Interactions between Animal Viruses

F. M. Burnet

Walter and Eliza Hall Institute, Melbourne

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Introduction

One way of looking at virus infection is to picture it as the intrusion of one genetic system (the virus) into the domain of another (the host cell) and the subsequent interactions of the two systems at various levels. An essential part of this concept is that of the eclipse phase during which the virus particle disappears as an individual and is represented, operationally speaking,

merely as the potentiality of the infected cell subsequently to give rise to a brood of descendant virus particles. There is an increasing volume of evidence mostly circumstantial, that the essential feature of this stage is the release of free nucleic acid from the virus particle and that the "information" needed for the production of descendant virus particles is carried at this stage wholly in the liberated nucleic acid. The inhibition of influenza virus multiplication by ribonuclease (Le Clerc, 1956; Burnet *et al.*, 1957) can probably be taken as evidence of such a phase. Recent findings that infective nucleic acid could be extracted from tissues infected with the viruses Mengo (Colter *et al.*, 1947), eastern equine encephalitis (EEE) (Wecker and Schafer, 1957), and Murray Valley encephalitis (MVE) (Ada and Anderson, 1957) provide strong support. Under such circumstances, it should be expected that if simultaneous infection of a cell by two related but distinguishable viruses can be effected, genetic interactions of one type or another will be likely to occur. This could be recognized by the isolation from the progeny of clones of virus manifesting a mosaic of characters, some corresponding to one parent, others to the second parent. Recent work indicates that such findings are by no means uncommon (Burnet and Lind, 1949). In this chapter, those aspects of the genetics of animal viruses which involve the appearance from double infections of progeny with characters derived from both parents will be considered. It will also be legitimate and convenient to include under genetic interactions those instances where a genetic character is, or appears to be, contributed by inactivated virus or some virus component, and to touch on the possibilities of multiplicity reactivation where the interaction of two or more "killed" virus particles allows the appearance of viable progeny.

II. The Different Types of Genetic Interaction

We may define our subject matter, then, as the analysis of those phenomena by which virus particles are produced which have observable characters derived from more than one parent form. The most important type of genetic interaction is that by which progeny can be isolated as a true-breeding clone with inheritable qualities derived from two (or more) parent forms. This will be referred to as *true recombination*. To date, the best examples have been observed with influenza viruses.

True recombinants have been obtained in which one of the parent strains has been inactivated as far as conventional tests are concerned by heat or ultraviolet light. It is therefore by no means inconceivable that agents equivalent to the transforming factors of bacterial genetics may be shown to exist. The Berry-Dedrick transformation of fibroma to myxoma is presumably of this character but has not yet been adequately analyzed.

Evidence has been provided by Gotlieb and Hirst (1954) that (presumably) unstable *heterozygotes* can be obtained. A heterozygote can be defined as a

single virus particle from whose progeny two or more genetically distinct and true breeding clones can be obtained. To satisfy this criterion experimentally, it is necessary to show that, from the population presumed to contain heterozygotes, an undue proportion of hosts receiving an average of less than one 50 % infective dose, allows the development of virus of two or more types. In practice, this has always meant the use of serological methods to detect the presence of two different parental serotypes in infective material from limiting infective dilutions.

When a double infection is produced with two different serological types of influenza virus in the allantoic cavity, the fluid obtained will often show the phenomenon of "double neutralization" of hemagglutinin. This indicates that the surface of individual particles must be made up of a mosaic of the two parental antigens. Hirst and Gotlieb (1953) use the term *phenotypic mixtures* for virus particles of this character. It is reasonable to believe that a large proportion of the viable particles in such populations are heterozygous, but since the two characters of double neutralization and heterozygosity are demonstrable respectively in large populations only and in individual infective units only, it is impossible to provide a rigid proof of this.

It seems highly probable that a fourth type of genetic interaction exists by which unstable forms emerge which cannot be maintained as clones of constant properties. This applies particularly to the virulence characters. When a new virulence is transferred by recombination to another serological subtype, it tends to be of lower level and more unstable than in the parent. We have therefore spoken of "*redistribution of virulence*" as something not quite the same as true recombination (Burnet and Lind, 1954b).

III. The Technical Requirements in Virus Genetics

A. Pure Clone Isolations

As in comparable work with bacteria, all genetic work with viruses should ideally be carried out with pure clones derived from a single parental particle. This is rendered almost impossible in all practical situations because of the fact that even with the best techniques for maintaining viability most of the viruses which have been studied show a significantly greater number of morphological particles than of viable units. With influenza viruses, standard allantoic fluids contain about 10 morphological to 1 infective unit (Donald and Isaacs, 1954). Horsfall (1954) contends that with adequate technical care the ratio can be reduced to unity. Even with the most elaborate care, polioviruses show a ratio of the order of 30–100 : 1 and with virus produced in standard fashion 1000–2000 morphological units may be present for each infective (plaque-forming) unit (Schwerdt and Fogh, 1957).

Two factors are probably responsible for these discrepancies: (1) For any given host-viruses system, the likelihood of any cell-virus particle interaction initiating demonstrable infection is always something less than unity. Where the host is of relatively low susceptibility, the proportion of fertile contacts may be very small. (2) Any population of virus particles will comprise some of intrinsically lower infectivity (cf. incomplete virus) and some whose infectivity has been reduced or destroyed by thermal degradation or other environmental influences.

Under the circumstances, it is not possible to provide any infallible rule for the production of pure clones of virus. If we could deal with a system in which virus particles were 100 % infective, the possibilities could be calculated. Theoretically, when equal numbers of infective particles A and B are mixed and inoculated into fully sensitive hosts at high dilution, one should obtain at the $1D_{50}$ level 17 % of positive harvests containing both A and B. At a further 10-fold dilution only 0.5 % will show both types.

In Liu and Henle's (1951) experiments, mixtures containing equal numbers of viable units of strains A and B were inoculated at various fractions of the $1D_{50}$ and the harvests tested by subinoculation in homologous antiserum for presence of the heterologous type. Their results gave about 30 % of double infections at the $1D_{50}$ level, which is nearly twice the theoretical expectation and suggests that, at some stage after infection has been initiated by a virus particle, cells still uninfected become more susceptible to infection by residual particles which otherwise would fail to initiate infection.

The practical difficulty in working with influenza viruses is, however, not quite so great as these figures suggest. Most of the double infections in Liu and Henle's experiments were only detectable by subinoculation in antiserum homologous to the apparent type of the harvested virus. There was a great excess of one form and a limit dilution of such a fluid would give a much lower proportion of double infections than is obtained from a starting mixture of equal parts of each virus. If we have a mixture in the proportion 90A : 10B, the theoretical proportion of double infections at the $1D_{50}$ level is reduced to 2.8 %. This principle can be made use of in practice by the *in vitro* characterization of all limit dilution fluids. If such tests give unequivocal results we can be certain that not more than 10–20 % of virus of different *in vitro* character is present. In any given experiment the likely types of "contaminant" will be known and the tests arranged accordingly. By using only fluids in which the type required is shown to be dominant by *in vitro* tests, the necessary procedures to obtain pure clone fluids are considerably reduced. We have found no exceptions to the rule that if two successive limit dilution titrations with 4–6 embryos per dilution each give three limit dilution fluids with uniform *in vitro* findings, all descendant clones are uniform in their *in vitro* qualities. Similar principles would presumably hold for any other virus

for which the limiting infective dose contained only a small number of morphological units. Much more difficult problems might be presented if genetic work were attempted with viruses which under optimal conditions showed 100 or more visible units per infective dose.

B. Marker Characteristics

In any study of genetic interaction, whether in viruses or in sexually reproducing higher organisms, the geneticists' method is to take as parents two varieties which (1) have sufficient common characteristics to allow the production of viable progeny and (2) differ preferably in two or more easily demonstrable qualities. The common characteristics can be taken for granted —it is the redistribution of differences among the progeny which is important.

An essential requirement for genetic work with viruses is therefore to have available strains differing from one another in easily recognized qualities but still close enough in other qualities to allow ready interaction. The available genetic markers, i.e., those characters for which differences can be readily shown, will differ from one group of viruses to another, and will be discussed in some detail (p. 284) for each of the three groups that have been studied.

At this point, it is only necessary to say something about three broad groups of qualities that can be used as markers.

1. *Morphological*

In general, recombination is not likely to occur between viruses of different morphology, but in the influenza viruses it can be shown (Burnet and Lind, 1957b) that the proportion of filamentous forms is an inheritable character of a strain and that this can be used in genetic studies.

2. *Somatic*

These are qualities depending on the nature of the free surface of the infective virus particles. They are specially important in the hemagglutinating viruses of the myxovirus group, simply because of the ease with which appropriate manipulations can be devised to manifest differences *in vitro*. A typical example is serological character, as demonstrated by hemagglutinin inhibition. When one of these qualities is demonstrated *in vitro*, whether by some modification of hemagglutination or by a complement fixation technique, we are concerned with the character of a large population of virus particles; the result observed will not be influenced if, say, 5 % of the particles are different in type from the majority. Only the nature of the dominant form will be recognized.

3. *Reproductive*

Somatic characteristics may remain unaltered in virus rendered nonviable by appropriate means. By reproductive characters we include the various manifestations of virulence—capacity to multiply in a given tissue and capacity to produce visible lesions or death. With some qualifications we may say that these require only the presence of a minimal infective dose in the inoculum to manifest the character in question, and that the presence of an excess of virus lacking the character will not, in general, prevent its expression.

Two qualities which may be needed as markers do not fall clearly into the reproductive group but must be considered along with them. These are "toxicity," in which lesions are produced without virus multiplication, and serological tests by neutralization techniques *in vivo*.

4. *Incidental*

For want of a better term this is used to signify those marker characters which depend on the production during the course of infection of a recognizable substance other than infective virus. The significant examples are vaccinia hemagglutinin and the "soluble" complement-fixing antigens produced by many viruses.

C. Principles of Recombination Experiments

Work on the genetics of animal viruses has necessarily been largely influenced by the methods which have been used with bacterial viruses. In both fields the ideal to be aimed at is to infect a population of uniformly susceptible cells in such a fashion as to ensure that the largest practicable fraction of cells are infected by at least one particle of each parental type. Any unabsorbed virus should be removed so that when the new brood of virus is liberated it should, as far as possible, be derived wholly from cells infected by the two parental viruses. The progeny should be harvested before there is any opportunity for a second cycle of infection to take place.

With influenza viruses, the allantoic cavity provides highly susceptible cells readily accessible to virus. These cells have been used for all work on recombination of influenza viruses, but other methods will obviously be required for other groups.

Fenner's finding that a high degree of recombination occurs where two developing pocks on the chorioallantois find newly proliferated cells in common may be a particularly satisfactory method when the main objective is to determine the qualitative range of recombinants that can be produced. With other types of virus, tissue culture methods will undoubtedly be the most satisfactory and there are obvious advantages for quantitative work in

using Dulbecco's monolayer culture techniques with viruses like poliovirus. It has not yet been reported that differences in plaque structures can be used as genetic markers but it seems highly probable that systems of this type will be found. It should then be possible to use the refined quantitative methods that have been used in phage genetics. At present, the necessity to isolate every clone to be characterized is a serious hindrance to satisfactory quantitative study. Whatever the technique of providing the conditions for interaction of two strains of virus, analysis of the results requires the isolation from the progeny of clones of virus among which recombinant characters can be detected. Ideally we should hope that, as is the case with most of the examples of genetic recombination that have been studied with bacterial viruses, the proportion of recombinants will be of the same order as of the parent types. This will allow isolation of recombinants without the necessity of providing a selective environment. There are, however, many examples where the yield of recombinants is much smaller, perhaps because recombination is only stable when one or other of the parents differs somewhat from the dominant form. In such circumstances, it will always be difficult to exclude the possibility that what is regarded as the recombinant is in fact a mutant. In most cases, a consideration of the characters of the new form against those of the two presumed parents will allow a tentative decision. Most such experiments will automatically include controls in which both parent types are also exposed alone to the selective environment.

Where experimentation is designed to elucidate the conditions under which recombination occurs, rather than to study the characters of the recombinant, it is desirable to use two parent strains which give rise to an easily recognized type of recombinant. The simplest such procedure, first demonstrated by Burnet and Lind (1954a), and also used by Baron and Jensen (1955), and by Hirst and Gotlieb (1956), is to use one inactivated parent with a serologically distinct active parent. After interaction the yield is subcultured in the presence of antiserum corresponding to the active parent. Any virus growing up and having the serological character of the inactivated parent is a recombinant.

Another method we have frequently used is to choose a pair Ab/aB which gives one recombinant AB which, when present in mixed culture, will overgrow ab, Ab, or aB. This allows recognition of the fact of recombination without the necessity of isolating and characterizing the recombinants.

In most situations of potential practical importance, the most important quality to be studied will be the virulence of strains and their mutants and recombinants. Virulence is notoriously labile when a virus is undergoing transfer from one type of host organism to another and some special requirements emerge in work on changes in virulence. In general the objective will be to allow a strain with a high virulence V for some defined host H to interact

with a strain with little or no virulence v for that host. The strains will also need to be differentiated by some other characteristic if results are to be intelligible, so that if this is differentiated as A and a, we have a virulent strain AV and an avirulent one av. Obviously, recombination experiments cannot be carried out in host H if we are to avoid the possibility of more virulent mutants of av being selectively favored. All such experiments require what can be called a neutral host (N) on which both virulent and avirulent strains can grow freely. In other words, both should be actively and, preferably, equally virulent for the neutral host. Fortunately, recent technical developments in virology have made it relatively easy to provide a neutral host in the form of an appropriate tissue culture or the chick embryo. If we are interested in mouse and monkey virulence of poliovirus, the neutral host will be a HeLa cell tissue culture, for mouse virulence of influenza viruses the allantoic cavity of the chick embryo, and for poxvirus studies the chorio-allantoic membrane.

If our experience with influenza viruses can be generalized, some special precautions are needed in characterizing recombinants. In many instances, when interaction occurs between virulent and avirulent strains and a number of LD fluids are tested at once for virulence, it is found that there is a considerable range of virulence. As a hypothetical example, suppose strain AV has virulence which can be given a value 8, while av for the same host has the value 0, after interaction, 10 LD fluids with the second character are found to have virulence against H of 0, 0, 0, 0, 1, 1, 2, 4, 4, 4. One of the fluids with virulence of level 4 is titrated and 10 LD fluids from this titration similarly tested against H. The result is likely to be 1, 1, 1, 1, 2, 2, 3, 4, 4. If one takes a primary fluid with virulence 1 and similarly prepares a set of descendant LD fluids the values will be perhaps 0, 0, 0, 0, 0, 0, 1, 1, 1, 1. In each case there is a downward drift in virulence, especially shown when we are concerned with the "transfer" of virulence to a strain with the other qualities of the avirulent parent. From the nature of the phenomenon it is extremely difficult to be sure that we are not dealing with mixtures in such experiments and it is always advisable to have a serological difference available by which at least one check for purity can be applied.

IV. Genetic Interactions within the Myxovirus Group

A. Historical

The first evidence that recombination could occur between animal viruses was published by Burnet and Lind in 1949. They were able to obtain from mouse brains inoculated with mixtures of a neurotropic influenza virus strain NWS (Stuart-Harris, 1939) and non-neurotropic strains of different serological

character, such as SW, strains in which neuropathogenicity was combined with the other serological type.

Owing to the striking and unusual character of neuropathogenicity in an influenza virus, a large proportion of subsequent work in our own and other laboratories has made use of a neuropathogenic WS strain as one "parent" in recombination experiments.

In Melbourne we found NWS not particularly suitable for work in chick embryos and changed in 1951 to the embryo-pathogenic strain WSE. With MEL/WSE mixtures a high yield of recombinants could be obtained either in intact embryos or in de-embryonated eggs. Extension of the work in various laboratories soon uncovered a variety of phenomena in addition to the transfer of neuropathogenicity from one serological type of virus to another. These include:

(1) The variable expression of neuropathogenicity in recombinants obtained from a neurotropic and a non-neurotropic strain (Burnet and Edney, 1951). In many such strains overt neuropathogenicity is shown only in mice less than a week old (Lind and Burnet, 1957b).

(2) The association of other changes with the transfer of neuropathogenicity (Burnet and Lind, 1951a) leading to development of the concept of two linkage groups in the MEL/WSE system (Burnet and Lind, 1952).

(3) The production of "doubly neutralized" hemagglutinin in the primary harvest of infections by mixtures of two serological types (Fraser, 1953; Hirst and Gotlieb, 1953).

(4) The demonstration of heterozygosity in virus showing double neutralization (Gotlieb and Hirst, 1954; Lind and Burnet, 1957a).

(5) The production of recombinants from mixtures of one active virus and another inactivated by heat or ultraviolet irradiation (Burnet and Lind, 1954a; Baron and Jensen, 1955).

(6) The observation of a variant in which the expression of several characters was prevented apparently by a suppressor or modifier gene (Lind and Burnet, 1958). To this should be added the finding that in the myxovirus group RNA is the only nucleic acid present in the infective particles (Ada and Perry, 1954; Zillig *et al.*, 1955). This makes the genetic behavior of influenza virus of great general interest, inasmuch as it is the *only* genetic system yet available for detailed study which cannot be based on a genetic code carried by DNA.

B. Available Markers in the Influenza Viruses

Genetic markers can be provided by any clearly demonstrable difference between two strains which are capable of genetic interaction. Those chosen will be determined essentially by the experience of the worker and the availability of techniques in the laboratory undertaking the investigation. Hirst and Gotlieb (1953) have virtually confined their attention to the two

sets of markers; the antigenic difference between WS and MEL and the difference between presence and absence of neuropathogenicity, as tested by intracerebral inoculation in 4-week mice. Baron and Jensen (1955) used in addition thermostability of hemagglutinin and pathogenicity for mice by the intranasal route. Burnet (1951) added pathogenicity for the chick embryo by the chorioallantoic route and the reaction of heated virus hemagglutinin with mucoprotein inhibitors.

In subsequent discussion of the phenomena of linkage of characters, most use will be made of the system based on the two influenza A viruses MEL and WSE and to some extent applicable to all influenza A strains that we have studied. These strains, as a result of a long series of passages since their isolation in 1935 and 1933, respectively, differed in at least six characters. It was convenient to represent the characters of MEL as ABCDEF and the corresponding alternative characters of WSE as abcdef. As these symbols have been widely used in our papers, it is desirable to provide some definition of each of the characters.

Aa. Serologically, as tested by hemagglutinin inhibition, the strains MEL and WSE have only a small common component and they can be easily differentiated with untreated antisera. By the use of appropriately absorbed sera the difference can be made absolute.

Bb. When MEL is heated at 56°C., there is little reduction in hemagglutinin titer. WSE titers are progressively reduced, usually to one-half or less after 30 minutes.

Cc. When certain strains of influenza virus are heated as crude allantoic fluid at 55–56°C. for 30 minutes, they become highly susceptible to inhibition of hemagglutination by a wide variety of mucoproteins, of which ovomucin and semipurified human meconium are convenient examples. This conversion to the "indicator state" character c is shown by WSE. MEL under the same conditions remains quite insusceptible to inhibition, character C.

Dd. This pair of characters similarly concerns the indicator state, but in regard to a single inhibitory mucoprotein obtained from sheep salivary glands. MEL cannot by any manipulation be made an indicator (character D) while WSE is converted by simple heating (d).

Ee. When WSE is inoculated on the chorioallantois of 12-day chick embryos, easily visible pocks are produced and the virus invades the embryo which is killed with gross hemorrhagic lesions in brain and other organs (character e). MEL produces no more than a light granularity on the membrane, rarely kills the embryo and produces no hemorrhagic lesions (E).

Ff. Strain WSE is highly pathogenic by intranasal inoculation in lightly anesthetized mice killing to a level of 1/10 AD (hemagglutinating dose). MEL given in standard dose of one hemagglutinating unit produces patches of nonlethal consolidation.

Where other strains than these present additional characters they are represented in similar fashion. For instance, two WS variants differ greatly in the heat stability of their hemagglutinin. WSM is not destroyed at 65°C., NWS is destroyed at 52°C. in 30 minutes. The qualities have been shown as B^+ and b^- respectively. Neuropathogenicity is symbolized by g, its absence by G. It must be stressed, however, that the symbols are primarily to represent phenotypic character differences between the two strains MEL and WSE. Any extension may lead to unjustified assumption that similar phenotypic characters are based on the same genetic mechanism as in the MEL/WSE system.

C. Interchange of Linkage Groups

Very large numbers of recombination experiments between MEL and WSE, and among various other strains, variants, and recombinants of influenza A types, have now been carried out. Examination of the recombinants shows that there is only a limited range of new patterns of characters and that with a few exceptions these patterns correspond to an interchange of only two linked groups of characters. This is most clearly seen from an analysis of the recombinants obtained from the interaction of MEL and WSE (Burnet and Lind, 1952):

$$\underset{\text{MEL}}{\text{ABDF} - \text{CE}} + \underset{\text{WSE}}{\text{abdf} - \text{ce}} \rightarrow \underset{\text{M}+}{\text{ABDF} - \text{ce}} + \underset{\text{WS}-}{\text{abdf} - \text{CE}}$$

The interaction is a reciprocal one. The primary recombinants (which we have called M + and WS —) can be repeatedly reisolated without change of character and are stable virus types. They interact to give strains of MEL and WSE character (Lind and Burnet, 1953):

$$\text{ABDF} - \text{ce} + \text{abdf} - \text{CE} \rightarrow \text{ABDF} - \text{CE} + \text{abdf} - \text{ce}$$

When a neuropathogenic strain is used the quality g is associated with the ce "linkage group," not with abdf. Neuro-MEL recombinants have the formula ABDF — ceg and when such a strain is crossed with WS — a neuro-WSE strain is obtained (Lind and Burnet, 1954):

$$\text{ABDF} - \text{ceg} + \text{abdf} - \text{CEG} \rightarrow \text{abdf} - \text{ceg}$$

Although the great majority of interactions follow this rule there are exceptions, e.g., in some cases the interaction of a strain of a different serological type CAM with WSE proceeded as follows:

$$\text{A}^c\text{DFCE} + \text{abdfce} \rightarrow \text{A}^c\text{D} - \text{fce} + \text{abd} - \text{FCE}$$

Here the character of mouse lung pathogenicity was in the second linkage group. From their limited tests it is probable that the strains Wr and WSN used by Baron and Jensen (1955) behaved similarly:

$$\text{Ab} - \text{FG} + \text{abfg} \rightarrow \text{Ab}^-\text{f(g)} + \text{ab} - \text{FG}$$

Since the only markers used in Hirst and Gotlieb's (1953) work were Aa and Gg, no evidence on this point is provided by their experiments.

D. Phenotypic Mixture and Heterozygosis

Early in the work on recombination between MEL and WSE strains in the allantoic cavity, it was observed that the primary yield of fluid often showed anomalous results when tested with antisera against the two parent strains. Some fluids contained what appeared to be a considerable proportion of hemagglutinin neutralized by both antisera. This was mentioned by Lind and Burnet (1953) and reported by Fraser (1953).

TABLE I

SEROLOGICAL QUALITIES OF X FLUIDS

Type of fluid	Neutralization *in vitro*	
	Anti-M	Anti-WS
M	32000	<32
W	<16	1500
X_1, X_2	2000	1500
X_3[a]	64	500

[a] The X_3 strain was significantly neutralized *in ovo* by both antisera.

A clearer picture of this phenomenon was provided from the experiments of Gotlieb and Hirst (1954), who were concerned with double infections by the strains MEL (M) and WSN (W). The latter strain resembles NWS of Stuart-Harris (1939) in general properties but was a separate derivative of WS obtained originally by Francis and Moore (1940). Gotlieb and Hirst (1954), using cross-absorbed immune sera, obtained from double infections a proportion of fluids whose HA was neutralized to considerable titer by both. These are called X_1 fluids. When such a fluid is titrated, both M and W type fluids are obtained among the progeny, as well as a proportion of X_1 fluids from embryos inoculated at low dilutions.

With continued titration passage of X type fluids, some were obtained which gave a much higher proportion of doubly neutralizable fluids, including some from dilutions near the limit of infectivity. These were referred to as X_2 fluids. They did not represent a stable form since they always gave a proportion of pure M and W fluids at limit dilutions.

Finally, an atypical fluid was obtained which, although predominantly W in type, was also significantly neutralized by anti-M serum. This bred true and was called X_3. The characters of these fluids are illustrated in Table I.

The chief interest of these results is in regard to the nature of the X_1 X_2 fluids. From the results obtained with antisera, it appears that perhaps 80 % of the virus particles have a mosaic surface of both antigens. This does not necessarily imply that 80 % of the *viable* particles have such mosaics, but the general correspondence of neutralization tests *in ovo* suggests that such is probably the case. One can see no *a priori* reason why viable and nonviable particles should not have the same type of antigenic surface structure.

In order to determine the *genetic* character of these mosaic particles, Gotlieb and Hirst (1954) carried out titrations, going well beyond the 50 % end point and analyzed the fluids so obtained for the presence of both serological types. They found a clear excess of fluids from which both M and W could be obtained in titrations of X fluids, as contrasted with mixtures of M and W. They believed that this was direct evidence of the existence of heterozygotes and, as will be discussed later, consider it likely that all recombination occurs via a heterozygote condition.

In our own experience (Lind and Burnet, 1957a), X fluids from limit dilutions are excessively rare in the systems we have used, and we have also found it much more difficult to obtain evidence of heterozygosis using the same methods as Hirst and Gotleib. On one occasion, a typical X fluid was obtained at an ID_{20} level from the primary harvest of a MEL/NWSE cross. This fluid A was analyzed in much detail by repeated titrations at LD by several methods. Direct titration showed LD fluids either WS — (a recombinant) or MEL in the ratio 17 to 7 (titers $10^{8.5}$ and $10^{8.1}$, respectively). Only 2 of these 24 clones showed evidence of being derived from a heterozygous particle. In addition, appropriate titration on the chorioallantois, with and without antiserum, gave invasive WSE and M + (recombinant) strains at titers $10^{5.6}$ and $10^{4.5}$, respectively.

Undoubtedly heterozygotes can occur —with important implications for the general understanding of virus genetics—but everything suggests that heterozygotes are less viable than homozygotes and that in Hirst and Gotlieb's system a much higher proportion of heterozygotes are viable for a few generations at least than in ours.

This is equally evident in their studies of recombinants from WSN (similar but not identical with NWS) and MEL which, using Hirst and Gotlieb's (1955) nomenclature, will be called W + and M —. Mixed cultures give harvests which on test at limit dilution give rise to three predominant types, the originals W + and M — and a W — recombinant. No directly neuropathogenic M + was obtained but a proportion of masked neurotropic M's could be detected by back-crossing with W —. The point of greatest interest is that if the clones serologically W were tested (1) for heterozygosity of the initiating virus by subculture in anti-W serum and (2) for + or — neuropathogenicity,

the results in Table II were obtained. The great bulk of recombinants was obtained from clones demonstrably heterozygous.

TABLE II

W Clones Obtained in Hirst and Gotlieb's (1955) Experiments

Clone	Homozygous	Heterozygous
W+	56	36
W−	2	41

In accord with this finding, when the primary harvest was titrated in the presence of anti-M serum, only W + clones were obtained. This suggests that heterozygotes with M components on their surface were neutralized and could not give rise to W — progeny.

The result differs from the fluid A mentioned above from Lind and Burnet's (1957a) paper. This gave virtually a full yield of the WS — recombinant when titrated in anti-MEL serum. The significance of these differences between the two sets of results are discussed later.

E. Use of Nonviable Virus as an Effective "Parent"

One of the most interesting features of genetic work with influenza viruses is the comparative ease with which recombinants can be obtained from inoculation, either as mixtures or in sequence, of one component killed by heat or ultraviolet irradation and the second in the form of active virus. This was reported for heat-killed virus by Burnet and Lind (1954a) and confirmed for ultraviolet-killed virus by Baron and Jensen (1955) and for both heat- and ultraviolet-killed virus by Hirst and Gotlieb (1956). In recent work (Lind and Burnet, 1957c), we have made a closer analysis of virus, heat-inactivated at various temperatures. The best yield of recombinants was obtained from fluids inactivated by slow thermal degradation at 37°C. for 20 days.

Nothing has been published to suggest that the type of recombinant obtained from such nonviable/viable crosses differs significantly from those from normal crosses. Burnet and Lind (1954a) found a considerably lower yield, but the types of virus obtained were of normal quality. Hirst and Gotlieb (1956) were more successful in obtaining M + with direct mouse pathogenicity from W +/M — crosses when the M — component was inactivated by ultraviolet light than when active virus was used. Baron and Jensen (1955) do not give data for comparable active/active crosses, but there is nothing to suggest that they would not have obtained similar recombinants.

For obvious reasons it is much simpler to obtain and isolate recombinants of the serological type of the inactivated parent than the reciprocal recombinant serologically similar to the active parent.

It is possible, however, to provide a selective environment for isolation of WSE recombinants from the system, heat-inactivated M +/active WS —, by inoculation of the chorioallantois and reisolation from the embryo lung. Two such strains were isolated and fully characterized by Lind and Burnet (1957c). One showed unduly low pathogenicity for the chick embryo.

All workers in this field have found that treatment of the allantoic cells with the inactive virus may be carried out one or more days before adding the active virus, and still give rise to typical recombinants. This observation seems to offer possibilities for analysis of the process of infection in the cell, but our own unpublished experience was that, although recombinants are regularly obtainable, the yield was very small and variable, so that the system we used was unsuitable for critical experiments.

A point of interest in the use of M + and WS — strains for crosses in which one component was inactivated at 37°C. is in regard to interference by the inactive virus given 18 hours before de-embryonation and addition of the active virus. Table III gives the findings of one set of experiments (Lind and Burnet, 1957c).

TABLE III

PRODUCTION OF RECOMBINANTS AND INTERFERENCE BY VIRUS INACTIVATED BY THERMAL DEGRADATION

Inactivated virus (37°C.)	Active virus	Interference [a]	Recombinant yield
M+	WS—	160,200/240	10^1
WS—	M+	<1/400	Nil

[a] Result shown as HA titer from combined infection/titer from infection with active virus alone.

In more extensive experiments a few WSE strains were isolated from heat-inactivated WS —/active M + systems. The suggestion that the process of interference prevents the genetic material being available for recombination might lead to experiments of some importance.

F. Redistribution of Virulence

Some discussion has been already offered in the general section of this contribution of the importance and difficulty of understanding the redistribution of virulence when virulent and avirulent strains are crossed.

1. *Transfer of Neuropathogenicity*

What can now almost be called the classic example is neuropathogenicity in influenza viruses. By using one or other of the available neuro-strains of WS (NWS—Stuart-Harris, 1939; WSN—Francis and Moore, 1940), it is possible to transfer neuropathogenicity to the influenza strains MEL (Burnet and Lind, 1951b; Burnet and Edney, 1951; Hirst and Gotlieb, 1953; Edney and Lim, 1954), Swine 15, WSM (Burnet and Lind, 1949), Oc. I. (Burnet and Lind, 1951b), KUNZ (Appleby, 1952), WSE (Lind and Burnet, 1954). By appropriate choice of recombinant strains, examples of all of these have been obtained with pathogenicity on intracerebral inoculation in mice approaching that of the original neuro-WS strain.

When, however, the whole yield of recombinants is considered, all workers find a high proportion of strains with low or equivocal neuropathogenicity.

Hirst and Gotlieb (1955) for instance, using W + and M — parents, found it easy to obtain W — recombinants but only 2/58 strains serologically M had potential neuropathogenicity. These were detected only by back-crossing with W — and obtaining neuropathogenic W + recombinants. Subsequent use of M — inactivated by ultraviolet light gave M + recombinants with full neuropathogenicity.

Fraser (1955) inoculated mixtures into the brains of infant mice and obtained a high yield both of neuro-MEL strains and the reciprocal non-neuropathogenic WS —. Like other workers he found that the neuro-MEL strains showed a wide range of expression in virulence by intracerebral inoculation.

In Melbourne, all our studies have shown a wide range of expression of neuropathogenicity in recombinants from NWS (Burnet and Edney, 1951). Whenever neuropathogenicity was evident in derivative strains serologically MEL, the two other characters of conversion to indicator and pathogenicity for chick embryos, later called c and e, were present. If, however, one took all strains with the complex of characters A-ce and tested their pathogenicity by intracerebral injection in mice, a complete range from no pathogenicity to a virulence corresponding to NWS was observed.

When recombination took place in the allantoic cavity between NWS and MEL, it was easy to obtain MEL strains A-ce but none of these was virulent for mice of standard size. Later work along somewhat different lines makes it likely that these strains would have been pathogenic for infant mice. Fully virulent neuro-MEL strains were only obtained when either the mouse brain or the chorioallantois was used as the route of inoculation. Fraser (1958) has succeeded in producing neuropathogenic recombinants in the allantoic cavity by repeated crossing of progeny showing A-c character on isolation, with the NWS parent. Ultimately, strains of overt but not maximal neuropathogenicity were obtained.

In some recent experiments (Lind and Burnet, 1957b), a recombinant NWSE of high but not completely regular pathogenicity for adult mice by the intracerebral route was used in crosses with MEL. Eighteen pure clones with M + *in vitro* characteristics gave almost uniform findings in regard to neuropathogenicity. In 4–5 weeks mice, inoculation of 1 AD intracerebrally gave only an occasional death but most mice appeared sick on the sixth or seventh day and virus could be isolated from the brain of those killed at that time. All were lethal to infant mice inoculated 2–4 days after birth. This type nM therefore appeared to represent a rather standard level of incomplete expression of neuropathogenicity. On back-crossing nM with WS —, the WSE type recombinants showed the same virtually stable low level of neuropathogenicity.

The interpretation of these results can hardly go further than an acceptance of the view that the fully neuropathogenic strains are the result of a series of genetic changes and that when the quality is transferred to other types a variety of relatively unstable forms emerge.

2. *Redistribution of Mouse Lung Virulence* (*MLV*)

In the system we have used in most work, MEL, WSE, and the recombinants M +, WS —, the stability of pathogenicity for the mouse lung and its consistent linkage with serological type has been most striking. Exceptions are so rare that one tends to regard them as due to contamination or technical error. All strains serologically MEL give lung lesions averaging 0.5–2.5 and never lethal after a dose at the 1 AD level; all strains serologically WS kill mice with complete consolidation at the 1 AD level and usually at one-tenth of that dose. This holds also when heat-inactivated components are used (Burnet and Lind, 1952, 1954a; Lind and Burnet, 1953, 1957c).

In these investigations, the interaction was between highly virulent WS strains and weakly virulent MEL strains. In an unpublished series of experiments a variant of WS — was obtained which was completely avirulent for the mouse lung. This allowed a cross between weakly virulent M + and avirulent WS — strains. The MEL recombinants still retained their standard weak virulence.

Mouse lung virulence is, however, by no means as stable in other systems. It is consistent with our experience to say that where linkage of mouse lung virulence with serological character is not evident one observes redistribution of virulence. The most interesting example concerns WSE which, as noted above, shows no evidence of instability of its high MLV (mouse lung virulence) in interactions with MEL. When crossed with CAM (no MLV) a proportion of recombinant CAM types showed moderate MLV; among WS — and WSE (isolated from the primary fluid) types there was a wide range of MLV (Burnet and Lind, 1955). Similar variability of MLV was observed in

recombinants with serological character PER in crosses between the recent filamentous strain PER (Persian Gulf 1/1951) and WSE (Burnet and Lind, 1956).

The papers dealing with these experiments were published before the findings of Briody *et al.* (1954) and Edney (1957) were available. These indicate that some strains, including CAM, may develop virulence for mice without experience of passage in the mouse lung. In Edney's work, adaptation to growth in the presence of anti-CAM serum in the allantoic cavity was associated with the appearance of a moderate degree of lesion-producing capacity. The possibility must therefore be kept in mind that in the WSE/CAM crosses the appearance of mouse pathogenicity in CAM + recombinants might have been an essentially accidental or mutational appearance in the course of the mutual adaptation of virus components in the new form. On the other hand, in this cross there was a significantly high yield of WS derivatives with MLV far below the normal level—types that were not seen in the MEL-WSE system.

In another type of experiment CAM was adapted to high MLV by serial passage in mice. The virulent strain CAM-MP was then crossed with a non-pathogenic derivative of CAM obtained by growth in anti-CAM serum and "marked" by serological resistance, CAM-SR. The progeny were sorted into serum resistant and serum sensitive clones and each tested for MLV. None of the SR clones had mouse pathogenicity but the sensitive clones showed a wide range of MLV from the original high level to zero (Burnet and Lind, 1954a).

Using an influenza B strain ROB adapted to mouse lung and crossing with the serologically distinct strain MIL, Ledinko (1955) obtained a similar range of lowered MLV in the derivatives of ROB serological type. In these experiments mouse lung pathogenicity was the only character showing transfer from one serological type to the other.

Precisely similar results had been obtained earlier in crosses between the influenza B strains LEE mouse pathogenic and MIL (nonpathogenic) (Perry *et al.*, 1954) and Table IV, modified from their paper, gives a good impression of the character of the redistribution of virulence of this type.

3. *Discontinuous Mutation Involving Virulence*

Brief mention should be made of two variant strains which we have recently studied in which there was a complete loss of virulence for the mouse. The most striking is a derivative from the highly mouse virulent strains WS —. Under appropriate allantoic passage at low dilution, reversion in stages to the mouse virulent form occurs and recombination between virulent and avirulent types gives some forms of intermediate virulence (Lind and Burnet, 1958).

The second example is a mutant of M + in which, in addition to loss of virulence for mouse and chick embryo, two other marker characters were changed. The strain could be passed indefinitely at limit dilution but rapidly reverted to normal M + on passage at low dilution. Recombinant experiments suggested that the types obtained were all derived by interaction of revertant M + with the other parent, WS − (Burnet and Lind, 1957a).

TABLE IV

DISTRIBUTION OF MOUSE LUNG VIRULENCE IN CLONES FROM THE INFLUENZA B STRAINS LEE AND MIL AND FROM FLUIDS RESULTING FROM THEIR INTERACTION IN DE-EMBRYONATED EGGS

Serology	Source	Grades of mouse lung virulence [a]				No. of clones
		0–0.25	0.3–1.1	1.2–2.4	2.5	
LEE	Stock	0	0	17	83	17
LEE	Exptl.	15	10	15	60	66
MIL	Stock	97	3	0	0	33
MIL	Exptl.	46	27	17	10	43

[a] Percentage of clones falling in each grade is shown.

G. Interpretation of Influenza Virus Recombination

Several attempts have been made to provide a general interpretation of the phenomena encountered in the field of influenza virus genetics (Burnet, 1957) but none has been satisfactory and it is conceivable that the difficulties may persist indefinitely. At this level of organization it is almost impossible to avoid confusion between phenotypic characters and conceptual genetic units or to be clear where chemical, physiological, or genetic concepts are needed. The difficulty extends to every aspect of the virus-host cell system and it is impossible to deal effectively with the genetic aspect unless an acceptable interpretation of the process of virus synthesis is available. This, of course, is not the case. If no comprehensive discussion is possible, the alternative is to look for the type of limited generalization that may be of value in the present stage of developing knowledge.

(a) It is clearly desirable to have available rules by which applications of virus genetics to matters of practical importance could be implemented. It has been suggested, for instance, that if a new serological type of influenza A should appear with exceptional human virulence of the 1918 type, it might be necessary to graft its serological character to another strain of high infectivity for human beings but low pathogenicity. Such a recombinant might allow the use of firebreak tactics to deal with a grave emergency. For purposes like this

all that is needed is a systematization of the results of recombination experiments to indicate the extent to which characters can be modified or recombined and the influence of experimental conditions on the results.

(b) Genetic information must obviously be considered in any attempt to provide a better picture of the processes taking place in the virus-infected cell. It may be of value to those interested in the broader problem to attempt to make clear some of the more immediate implications of the genetic findings.

1. *Genetic Aspects*

At the genetic level the main findings have already been outlined. Points which may be stressed are as follows:

(a) The existence of heterozygotes indicated that single infective particles may contain two or more sets of genetic determinants (genomes).

(b) Phenotypic mixtures of serotypes is a regular phenomenon, presumably indicating that in the doubly infected cell there is no dominance of one set of genomes over another.

(c) Recombination is limited to interchange of two groups of characters; hence no more than two genetic loci can be postulated. On the other hand, characters within a single group can readily undergo mutational change independently.

(d) In our hands, heterozygotes are rapidly replaced by homozygotes.

(e) The behavior of virulence in genetic interactions is complex. In some examples, crosses between virulent and avirulent forms result in the appearance of clones with a wide range of intermediate virulence. In other systems, and particularly where a virulence character is linked to serological type, no such redistribution of virulence occurs.

Before discussing these points it is advisable to make some comment on the symbols used. We believe that the most convenient convention is to retain our present nomenclature for the phenotypic differences initially between MEL and WSE using Roman letters; and to apply these if necessary with distinguishing superscripts to phenotypic characters used in work with other strains. Where discussion of genotype is required we believe that until an accepted basis of theory is available the same symbols should be used in italic script. In the case of MEL, the genotype will be *ABDF-CE* without prejudice to future decision as to whether *ABDF* represents one genetic locus or many. It is believed that this set of symbols could be modified to deal with new facts and ideas as they developed.

a. Heterozygosis. If one takes account of the findings in regard to heterozygosis in both Hirst's laboratory and our own, certain likely conclusions can be reached. Our results with the MEL/WSE system show that when fluids are initiated with single infective units from the primary harvest of a recombination experiment with a high level of phenotypic mixture, the great

majority are serologically homozygous. If most viable particles showing phenotypic mixtures are heterozygotes, as Hirst and Gotlieb's experiments suggest, some means must be available by which infection with a heterozygote gives a homozygous yield. An obvious suggestion from analogy with what is known from work with bacterial viruses is that the initiation of replication by one virus genome inhibits replication by any other type of genome which may also be present. The existence of recombination in itself makes this impossible or highly unlikely. The simplest interpretation is probably to make the assumption that the chance of *any* genome initiating multiplication is quite low and that the virus particle contains *n* genomes to ensure the likelihood of one being effective. In line with this is Donald and Isaacs' (1954) finding that in the average fluid only 1 of 10 visible particles initiates infection. This provides a simple explanation for the difference between American and Australian results. In our system, slightly less than 1/n genomes succeed in initiating infection, so that virtually all yields are homozygous, in Hirst and Gotlieb's system about 2/n succeed and heterozygotes are frequent. Other possible explanations are more conveniently deferred till the relation between genetic changes and virus multiplication is discussed.

b. Virulence. Virulence in a virus is necessarily a phenomenon of multiple causation. If we consider a virus of maximal virulence for the standard host as the normal form, then any genetic change which reduces the effectiveness of its functioning will diminish virulence. To be recognized as virulent an influenza virus particle must be able (1) to attach itself to and enter the host cell, and (2) to bring viral components not concerned with (1) into necessary relationships with synthetic mechanisms in the cell. When a virus is transferred to a new host, many modifications will be necessary in both respects before it can be brought to maximal virulence; all detailed studies indicate that any such development of a new virulence is the end result of many small mutational changes, probably involving nearly all genetic determinants.

With this background we can consider the influence of genetic interaction on virulence in typical systems, concentrating particularly on the interaction between the neuropathogenic NWS and MEL. Here the usual result is recombination to give ABDF-ce (g) (neuro-MEL), but in the allantoic cavity the recombinant has not in our experience been able to kill 4–6 week mice inoculated intracerebrally (Fraser and Burnet, 1952). To obtain mouse virulent forms the interaction must take place in the mouse brain or within the tissues of the chick embryo. There is also very considerable variability in the manifestation of neuropathogenicity among replicate limit dilution fluids. There are two possible approaches. One is to avoid any attempt to provide a detailed genetic interpretation by recognizing that the new complex *ABDF-ceg* (the symbols being italicized to indicate that we are dealing with the genetic determinants that were responsible for the phenotypic characters

of MEL and NWS) is a new and untried combination that is only going to "work" adequately when many minor mutations and perhaps recombinations are sorted out by survival in an appropriate environment.

The second would be to look at the process by which neuropathogenicity developed as one demanding a series of mutations at several loci, so that the difference between G and g might be expressed as $G^1\ G^2\ G^3\ G^4\ G^5$ and $g^1\ g^2\ g^3\ g^4\ g^5$. If in the replicating pool of the doubly infected cell genomes carrying these alleles underwent a series of matings in the special sense used by Levinthal (1954) for phage, and were repeatedly subject to selection for survival, both in the intracellular pool and in the intercellular phase as the virus particle, then we might expect the emergence of some such complex as $g^1\ G^2\ g^3\ G^4\ G^5$ in the neuro-MEL form best fitted to survive. In essence, the second suggestion is only an attempt to visualize one way in which the requirements of the first might be provided in conventional genetic terms.

From the more general point of view, we can also consider the reasons why, when a virulent and an avirulent strain are crossed, the commonest finding is an asymmetrical redistribution. If we have two serotypes P and Q, with virulence expressible as 4 and 0, respectively, then the progeny from recombination will show most Q serotypes avirulent, with only a small proportion representing low to moderate, grades 1 and 2, virulence.

The P serotypes corresponding to the virulent parent will, on the other hand, show a larger proportion of P serotypes, with diminished virulence running right down to zero (Burnet and Lind, 1954c).

It is of special interest that in the system which does not show redistribution of virulence (MEL/WSE), mixed infection gives a high proportion of recombinants which can be isolated without recourse to selective processes. With a very similar system, CAM/WSE, in which only a small yield of recombinants can be obtained, there was evidence that redistribution of mouse lung virulence could occur (Burnet and Lind, 1955). This was expressed as:

$$\frac{A^cDF^-\text{-}CE \times adf\text{-}ce}{A^cDFcE,\ A^cD\text{-}fce,\ ad\text{-}FCE,\ adf\text{-}CE}$$

In other words, the redistribution of mouse and chick pathogenicity on the recombinants characterized *in vitro* as A^c-c or a-C could take almost any form. The only limitation was that the virulence of the derivative was always lower than that of the parent.

All these findings fit into the general pattern that virulence for the mouse lung is dependent on a delicate harmony within the genotype. While it is in process of being established by passage or when recombination is occurring, deviations are possible which will sharply reduce the virulence. At any recombination all the progeny of doubly infected cells will probably contain some mixture of genetic elements, and even those which superficially retain

the genotype of the virulent parent will have had to make rearrangements which do not allow such smooth functioning as the parent. Hence, a lower virulence is likely except in the circumstances already mentioned of the MEL/WSE system, where mutual adaptation seems to be particularly easy.

With a recombinant of low virulence, i.e., by hypothesis, one in which the genotype is lacking in mutual adaptation of its components, is passed in the allantoic cavity at limit dilution, the almost invariable finding is a further loss of mouse virulence. Adaptation by minor mutation or any other mechanism is to the conditions of multiplication in the allantoic cavity, not in the mouse lung. Any change will, therefore, tend to deviate the genetic structure away from mouse virulence.

c. Genetic loci. The above discussion of virulence also has some bearing on the number of genetic loci that may be postulated. The very conservative view from the MEL/WSE system that there are only two loci could well depend on the greater predominance of recombinants of the types found. Even if small numbers of every other possible recombination of our six marker characteristics arose, they could not be detected by current techniques. The CAM results have other possible interpretations but they are compatible with a genetic structure involving a large number of allele differences but very limited capacity to produce viable recombinations of any sort. Those that were produced and isolated may well have depended on minor preliminary mutation in one or the other component; the chief difficulty in interpreting the results of any recombination experiment giving very small yields of progeny combining parent characteristics is to exclude the possibility that the changes are due to simple mutation and a complex environment where interference or other conditions in doubly infected cells may provide unusual criteria for selective survival.

The crux of the matter is that no detailed genetic interpretation is possible without extensive numerical data which, with current techniques, could only be provided for the dominant recombinants in a few specially favorable systems. Where rare types have to be obtained by the use of selective environments, it is hardly possible, even in principle, to obtain more than qualitative information. Such information, however, could be valuable in providing evidence at least of the number of loci that can be demonstrated and should be sought by the use of a wider range of selective environments.

2. *The Bearing of Genetic Findings on the Nature of Influenza Virus Multiplication*

It is a truism that between the inoculation of a single infective particle and the harvesting of the virus yield from an infected organ or cavity, a dozen virus-cell generations and an intense struggle for survival have taken place. The harvest undoubtedly contains mutant and incomplete forms, most of

which, because of their smaller number and lack of qualities for differential survival, cannot be obtained for study. The process of selective survival takes place at two levels, among virus particles in the stage between maturation in one cell and initiation of infection in another, and in the intervening processes which take place in what we have called the replicating pool of the host cell. At the level of the virus particle, one can imagine that the qualities making for selective survival concern mostly (1) resistance to nonspecific thermal degradation, (2) the effectiveness in relation to host cell surface components of the adsorptive-enzymatic structures of the virus surface. Some direct evidence in favor of the second viewpoint was given by Stone (1951).

At the intracellular level the types of competition and interaction among multiple genomes can only be envisaged in speculative fashion. From our own experience, we believe, as indicated earlier, that the crucial finding is that homozygous virus particles free from any association with serologically heterotypic virus are regularly obtainable in recombination experiments. Under the conditions of Hirst and Gotlieb's experiments, however, equally convincing evidence was obtained that heterozygosis is a necessary preliminary to recombination.

There seem to be three ways, not necessarily mutually exclusive, of picturing what happens when multiple genomes, either from a single virus particle or from more than one virus particle, enter a susceptible cell. In view of the existence of heterozygotes, we shall make the initial assumption that in a viable influenza virus particle there are n complete genomes (n being a small number, 2 or greater, and not necessarily uniform for all virus particles in a population).

The first alternative that only a small proportion of the genomes entering the cell can initiate replication has already been discussed.

The second possibility is based on the assumption of a relation to the phenomenon of incompleteness. There is still controversy about the nature of incompleteness but one can feel confident that ultimately it will be related to the conditions for the selective survival of multiple virus genomes in the cell. The emergence of a homozygote may mean that when two dissimilar genomes enter a cell and one for any reason initiates infection significantly before the other, the first clone will succeed in providing genomes for the new generation of fully viable virus, the second being responsible only for incomplete virus.

The third approach is based on a particular interpretation of how the new virus particles are fabricated at the free surface of the host cell (Burnet, 1956). It was held that the phenomena of phenotypic mixture plus a variety of other evidence indicated that the structure of the virus surface is derived rather directly from the surface membrane of the host cell. At the time in question it is assumed that the lipids and mucopolysaccharides of the cell surface are reinforced with specific viral protein produced in the replicating pool. If more

than one type of viral protein is being synthesized, the units will be distributed at random in the surface membrane in proportion to the rates at which the different types are being produced.

There is much more doubt about how the genetic material (assumed to be the central core of RNA nucleoprotein) is assembled. One way of looking at the process is to think of each genome as comprising two nucleoprotein units corresponding to *ABDF* and *CEG* genetic units. It may be that an aggregation of nucleoprotein, including appropriate numbers of both genetic units to give *n* genomes, forms just under the cell surface. We have been inclined to think of this as a random aggregation of genic material but the third way of deriving homozygotes from a heterozygous input would be to assume that only homologous genomes can be included together if a satisfactory viable "nucleus" is to be produced. On the whole, however, we should still prefer the view that the nuclear material of the virus particle is a loose aggregation of approximately constant genetic composition built up by random selection of what is available in the replicating pool.

V. Genetic Interaction in other Virus Groups

A. Recombination with Vaccinia and Rabbitpox Strains

Typical recombination of genetic characters can be observed in the poxvirus group. Work by Fenner (1958), and Fenner and Comben (1958) has been presented at laboratory meetings in Australia and I am indebted to Professor Fenner for permission to quote some unpublished work from his group.

Strains of variola, vaccinia, rabbitpox, cowpox, and mousepox show close serological interrelationships (Downie and Dumbell, 1956) and can be regarded as a natural group (Fenner and Burnet, 1957). Omitting mousepox and variola strains, Fenner (1958) has made a detailed study of 24 strains from the point of view of obtaining strains carrying suitable markers for genetic experiments. These could be placed in four categories:

(1) Standard dermal strains of vaccinia virus.

(2) Neurovaccinia adapted to intracerebral passage in the rabbit and the very similar strains isolated from rabbitpox epizootics.

(3) Jennerian cowpox strains.

(4) "White" variants especially characteristic of cowpox but also obtainable from neurovaccinia and rabbitpox strains. These give opaque white pocks on the chorioallantois (Downie and Haddock, 1952; van Tongeren, 1952).

The following characters were adopted as markers after experience had shown that they gave reproducible results with parallel clones and could be readily used for screening tests:

(1) Morphology of pock on the chorioallantois (CAM).

(2) Production of hemagglutinin (HA).

(3) Heat stability as tested by 40 minutes heating at 55°C. and subsequent titration on the chorioallantois (HR).

(4) Virulence for mice on intracerebral injection of a standard dose of 10^{4-5} pock-forming units (MV).

(5) Character of lesion produced by intradermal inoculation of a similar dose in the rabbit skin (RS). Of the strains available the most suitable pair for recombination work were judged to be a rabbitpox strain from Holland RP and a "white" vaccinia variant 7N. Their differences are as shown in Table V.

TABLE V

MARKER CHARACTERS USED IN POXVIRUS RECOMBINATION EXPERIMENTS

Character	7N "white" strain	RP (rabbit pox)
CAM	Large opaque white pocks (W)	Thin rimmed pocks with central hemorrhagic area (H)
HA	Produced in large amount (+++)	No hemagglutinin produced and no anti-hemagglutinin in serum after recovery (O)
HR	Labile: showing a fall of 4–5 log 10 on heating (L)	Stable: 1 log 10 or less (S)
MV	Minimal 4 % of deaths (—)	100 % deaths (V)
RS	Pink or red papular lesion without necrosis (N)	Flatter lesion with necrotic purple centre (PC)

Various types of double infection have been used. The best yield of recombinant forms was obtained from membranes inoculated with about equal numbers of both types, on which there was an example of pocks of the two parent types overlapping. This common zone will presumably include many cells which have suffered multiple infection by both types of virus. The area was cut out, emulsified, and titrated on the chorioallantois. More recently positive results have been obtained from double infections produced in HeLa cell tissue cultures. For the experiments so far completed only white pock clones have been studied for evidence of recombination. The recombinants reported can be represented as in Table VI, where 0 represents the character of the white vaccinia parent, + that of the rabbitpox strain and ± an intermediate result.

Examination of the table suggests no linkage between any of the characters except perhaps between the two virulence markers, where it is by no means complete. Perhaps the most interesting feature is the fact that when either virulence is present it is in all instances of intermediate character. This is

in line with the difficulty of "transferring" virulence in influenza virus recombinations.

As yet these results do little more than establish the fact that typical recombination of characters can be observed in this group of viruses. There is still need of a more controllable means of inducing double infection of cells which will allow analysis of first-cycle yields without the complication of selective factors which must be present in the overlapping area of two dissimilar pocks. Fenner is hopeful that this may be found by the use of HeLa cells in tissue culture.

TABLE VI

TWELVE RECOMBINANT CLONES FROM MIXED INFECTIONS WITH VACCINIA 7N 00000 AND RP (RABBITPOX) +++++

CAM	HA	HR	MV	RS
0	+	0	0	0
0	+	0	0	0
0	0	+	0	0
0	0	+	0	0
0	0	+	0	0
0	0	0	±	±
±	+	0	0	0
0	0	+	0	±
0	0	+	±	±
0	+	+	0	0
±	+	+	±	0
0	+	+	±	±

B. Interactions among Intestinal Viruses

The position in regard to the polioviruses is still far from clear and so far only preliminary reports have appeared. There seems to be no doubt that when two serotypes are grown together in tissue culture the harvest differs from a simple mixture of the same serotypes grown separately. An unduly large proportion of the plaques do not contain one of the normal serotypes. Some contain both serotypes, others contain doubly neutralized virus (Sprunt *et al.*, 1955; Black and Melnick, 1956). Similar findings have been reported for cultures from mixed infections by ECHO1 and polio 1 viruses by Benyesh *et al.* (1957) from Melnick's laboratory. The significance of these results is far from clear, but there is a distinct resemblance to the influenza virus findings. A *prima facie* interpretation would be that the standard virus particle is built up of a small number of subunits (as suggested by Crick and Watson (1957) and Caspar (1957)), each perhaps equivalent to a single genome. If these morphological subunits each carrying

the serological character of its type are to some extent interchangeable between related viruses, but as would be expected the "homozygous" complex has a survival advantage over others, then we have the basis for a reasonable interpretation of the findings. What is needed is a prolonged series of passages of doubly neutralized material to obtain a stable serological recombinant or to prove that it is impossible.

The alternative interpretation has been proposed that the essential effect is merely aggregation of virus particles of the two types. Reasonable *ad hoc* assumptions that this is more likely to occur in a doubly infected cell than in a simple mixture and that neutralization of one component may have an effect of interference or inhibition on the other when the complex enters a cell, would allow an equally satisfactory explanation.

As in the case of influenza virus, more meaningful results may be obtainable if markers other than serological ones can be utilized. A start in this direction has been made by Vogt *et al.* (1957) who obtained *d* (delayed) mutants of a type 1 poliovirus. These have lower pathogenicity for the monkey and on slightly acid pH 6.8 medium show a marked delay in the appearance of plaques on monkey kidney monolayers. Another heat-resistant mutant type has been obtained, t_1 t_2, which on crossing with the wild type gives clones of intermediate heat resistance (Dulbecco, 1957). Further reports of this work are awaited with much interest.

C. Genetic Interaction with Psittacosis Virus

Gordon and Mamay (1957) have demonstrated interaction between two strains of psittacosis virus marked by specific drug resistances. One obtained by Golub (1948) was resistant to sulfadiazine SA-r, the second to chlortetracycline Ctc-r. Each was fully susceptible to the other drug. The experimental method was to grow a mixture of SA-r and Ctc-r in embryonated eggs protected by *both* drugs in amount adequate to protect against either strain alone. The embryos were protected beyond the usual period but late deaths occurred and from these doubly resistant clones of virus were regularly isolated. Since the development of drug tolerance normally requires a prolonged series of passages, the combination of the two types of drug resistance after a single passage points strongly to genetic interaction.

D. The Berry-Dedrick Phenomenon—Transformation of Shope Fibroma Virus to Myxoma Virus

In 1936, Berry and Dedrick described a phenomenon which probably represents the first instance of genetic interaction between two viruses. Their experiments were stimulated by those of Griffiths (1928) on the transformation of pneumococcal type by inoculation of mice with a live R strain of one

type and heat-killed smooth organisms of another. The analogous experiment was to equate fibroma virus with the R pneumococcus and myxoma virus with the more virulent smooth form. Mixtures of extracts from fibroma and myxoma lesions were prepared, in which the fibroma virus was untreated and the myxoma extract heated to a temperature, which might be anywhere between 56 and 85°C. (Berry, 1937), that completely inactivated it. A substantial proportion of such inoculated rabbits developed myxoma lesions.

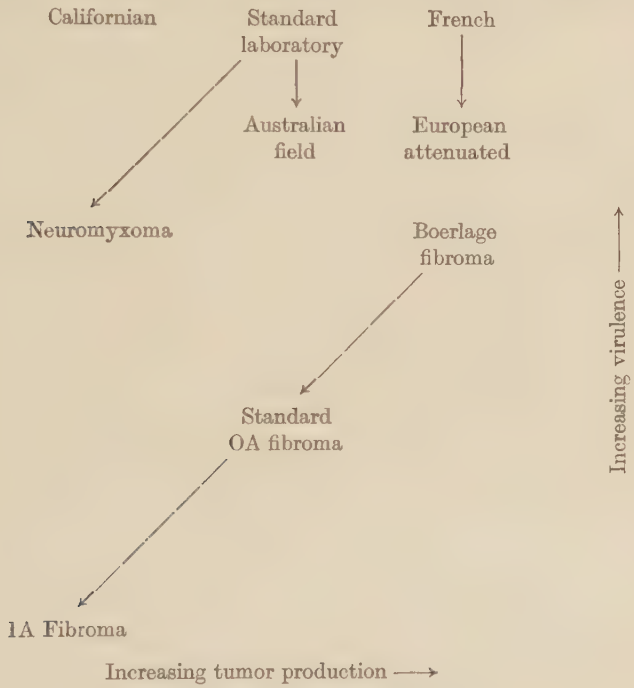

Fig. 1. Myxoma-fibroma relationship.

This observation has been repeatedly confirmed (Gardner and Hyde, 1942; Shope, 1950; Hurst, 1937; Smith, 1952) but all workers have found exasperating irregularities in the results.

The most extensive recent account is that of Smith (1952). She points out that the viruses of the myxoma-fibroma group show a wide range of virulence and a tendency for dissociation of tumor-producing capacity and virulence. If we combine Fenner and Marshall's (1957) subsequent Australian studies, we can present a two-dimensional arrangement of strains according to tumor-producing power and virulence (Fig. 1). A particularly interesting point is that in the fibroma series at least prolonged storage in glycerol produces an inheritable downward shift in the type of pathogenic activity, the end point

being Andrewes' (1936) inflammatory 1A variant, which produces only a mild inflammatory lesion in the rabbit (Andrewes and Shope, 1936).

Smith finds that transforming agents TA (i.e., heated extracts of infected tissues) prepared from strains of any type will (with some irregularity) allow transformation of strains lower in the series up to a level not higher than that of the virus from which the agent was prepared. If a low grade fibroma approaching 1A in character is used with myxoma TA, the usual result is not the production of myxoma but of a more active fibroma resembling the Boerlage type (Shope, 1950). Similarly, the inflammatory variant can be converted to standard fibroma by treatment with Boerlage fibroma TA. The transformed strains breed true on passage. Even where typical myxoma is produced the usual finding is that the rabbit inoculated with the primary mixture produces a large tumor which eventually regresses. Rabbits subinoculated from this lesion develop typical lethal myxomatosis.

The transformation has not been produced in experiments using the chorioallantois (Hoffstadt and Pilcher, 1941; Smith 1952), but Kilham (1956, 1957) has recently described successful transformations in experiments with tissue culture growth of the viruses.

In discussing the conditions under which successful transformation can be obtained, Smith (1952) notes individual variability in rabbits but could not relate this to such factors as age, breed, and sex. The transforming agent seemed to be the least variable component of the system. This can be prepared from any tissue containing adequate amounts of myxoma virus. The temperature of inactivation is not critical and may be as high as 85°C. and still give active material (Berry, 1937). As would be expected, it remains active in sealed ampules indefinitely at refrigerator temperature. The only evidence as to its chemical nature is found in the fact that active solutions can be deproteinized by repeated treatment with chloroform without loss of activity (Smith, 1952). This, plus resistance to treatment with alcohol, is at least consistent with the assumption that the agent is a nucleic acid, but does not exclude other possibilities, e.g., mucopolysaccharide.

It is to be hoped that a full analysis of this phenomenon will be forthcoming in the near future. Present evidence does not seem adequate to accept it as a transformation analogous to the pneumococcal transformation although, of course, this interpretation is not excluded. If nucleic acid is concerned it is most desirable that it should be identified as DNA or RNA.

Perhaps the most interesting feature is the observed capacity of storage in glycerol to "enforce" an inheritable change to a lower level of activity. This change can only be reversed by the use of transforming agent from a form higher in the series. There is a suggestion here that something other than a straightforward survival of a glycerol-resistant mutant is taking place. The evidence does not allow any specific suggestions, but one would feel that it is

highly desirable that limiting dilution isolations of virus should be made at various phases of the process. This should be possible by the methods used by Kilham; without it no reasonable understanding of the phenomenon is possible.

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Chapter XII

Problems Concerning the Tumor Viruses

Howard B. Andervont

National Cancer Institute, National Institutes of Health, Bethesda, Maryland

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I. Introduction

The idea that viruses could be implicated in the origin of tumors was expressed by various investigators, especially by Borrel (1903) in connection with his studies of other viruses. He was impressed with the number of viruses that elicited hyperplastic reactions in infected tissues and drew the inference that a continuation of this proliferative property could result in malignancy. His suggestions were justified for, within a few years, viruses were implicated in the origin of several tumors of chickens and, subsequently, in tumors of rabbits, frogs, and mice.

It is now known that tumor viruses may have insect vectors, may be transmitted by way of the excreta, the egg, or in mother's milk. They are not

only transmissible to individuals of contemporary generations, but are transferred to successive generations.

The tumor viruses are unique in that their activity or growth is adjusted to the host cell in such a manner that they modify the cell without destroying it. They are capable of inciting pronounced alterations in their host cells which enable the cells to ignore or to overcome the normal processes of control. And some tumor viruses, in contrast to the slow response to chemical carcinogens, produce this permanent change within a few days.

To those interested in the biological approach to the cancer problem the virus theory has always been of considerable interest and it has never lacked the vigorous support of a few investigators.

Extensive reviews on the subject have been published within the past five years. These reviews (Duran-Reynals, 1953; Oberling and Guérin, 1954; Dmochowski, 1957) and other publications (Rous, 1936, 1946; Andrewes, 1950, 1953; Stanley, 1957; Annals New York Academy of Sciences, 1952, 1957; Texas Reports on Biology and Medicine, 1957) set forth clearly supporting evidence for the idea that viruses could be etiological agents of cancer and the progress attained in the studies of known tumor viruses. In view of the excellence and inclusiveness of these presentations it is unnecessary at this time to prepare another comprehensive review. Instead, a few known tumor viruses have been selected for a discussion of their properties and to present briefly the major contributions and problems with which they are associated.

II. Problems Reviewed

A. Visceral Lymphomatosis of Chickens

The virus of visceral lymphomatosis deserves first mention because fowl leukemia was the first tumor shown to be caused by a virus (Ellerman and Bang, 1908). Years of research (Engelbreth-Holm, 1942) revealed that the etiological factor consisted of a group of viruses, each of which elicited a characteristic syndrome (Foulds, 1934). The virus of visceral lymphomatosis serves here as a representative member of the group because much is known of its biological properties and its routes of transmission.

Knowledge of the relationships between the virus and its hosts was acquired through years of patient investigation and is of considerable importance to the entire field of cancer research (Burmester, 1957). Chicks were found to be far more susceptible than adult chickens. Burmester and Denington (1947) used 1-day-old chicks in their successful transmission experiments with cell-free extracts of the naturally occurring disease. Burmester (1952) inoculated 5 age groups of chickens with a filtrate from a transplantable strain of visceral lymphomatosis and in doses proportional to

the size of the animals; the incidence of resultant tumors was 95 % in 2-day-old hosts but only 31 % in 114-day-old animals. It was also established that chicks were far more susceptible than adults when exposed to natural infection through contact with infected birds. Of paramount importance to the problems of tumor viruses was the finding that infected birds did not develop the disease until months after they had acquired the virus and many lived their life spans without revealing signs of the disease. The ability to parasitize without producing overt disease is also a characteristic of other tumor viruses.

Genetic factors were found to be of prime importance in determining the host's degree of susceptibility to the virus of visceral lymphomatosis. Waters (1945, 1951) has developed inbred lines of chickens showing pronounced variations in their susceptibilities to the virus when it is transmitted under natural or artificial conditions. The importance of the host's genetic constitution in problems of the tumor viruses will be emphasized throughout this chapter.

Efforts to acquire knowledge of the epidemiological factors concerned with the spread of the disease have supplied further information of host-virus relationships as well as a fascinating story in virus transmission (Burmester, 1957). Exposure of normal chicks to infected chicks resulted in the former showing a high incidence of the disease, and this was explained by detecting the virus in the debris of incubators. Succeeding experiments revealed the virus in feces and oral washings and showed that the saliva of chicks became highly infectious soon after exposure to the virus. These findings established the routes of virus transmission as contaminated food and water when the young birds were kept in close contact in crowded pens.

Meanwhile, some workers had observed the occurrence of the disease in chicks hatched from eggs in areas where chickens had never been kept and in others hatched in germ-free incubators. Such observations strongly suggested transmission of the virus through the egg and direct evidence to this end was obtained when the virus was found in livers from chick embryos. Egg transmission has been studied intensively and, among other findings, it was shown that the virus is more prevalent in eggs laid by hens from an inbred stock susceptible to the disease than by hens belonging to a resistant stock. Further, more virus was in the eggs laid by hens one year of age than in those laid by the same hens one year later.

Egg transmission was used to further knowledge of host-virus relationships. Progeny were hatched from eggs of birds known to shed virus in their eggs and these progeny also laid eggs which contained the virus. Throughout the experiments the hens remained in good health during the egg-laying period and chicks from infected eggs developed normally. Thus, the virus persisted in hen, egg, embryo, and chick without exerting any untoward effect upon

any of its hosts. Virologists will recall that a similar condition was encountered in a mouse colony carrying the virus of lymphocytic choriomeningitis (Traub, 1939).

Further studies established the genetic constitution and age of the hens as important factors in controlling the amount of virus shed in eggs and that the propagation of the virus in infected eggs was dependent largely upon the quantity of virus the egg received from the dam. When progeny of hens, known to be heavy, light, or non-shedders of virus, were observed for the occurrence of visceral lymphomatosis, many progeny of heavy shedders did not develop the disease. This result emphasized that maternal antibodies from infected dams were also deposited in their eggs (Andrewes, 1939) and protected the chicks during the critical early period after hatching. Neutralizing antibodies were found in day-old chicks from eggs of heavy virus-shedding hens, and, following this lead, it was observed that progeny of immunized hens were much more resistant to infection than those hatched by the same hens before they were immunized. Thus, this series of studies led to hopes of practical control measures.

It is clear that, fifty years after the discovery of the fowl leukoses viruses, one member of the complex is yielding to persistent efforts. Viewed in retrospect, it is surprising so much time was required to arrive at the present state of knowledge, which holds promise of preventive measures for control of the disease. Burmester (1957) explained this in his recent review. There are three forms of chicken lymphomatosis: ocular, neural, and visceral, depending upon the type and location of the predominating lesion, but two or all three forms may be present at the same time in a flock of chickens, or in one member of a flock. In several countries the neural and visceral forms were recognized more than fifty years ago, but during the first thirty years neural lymphomatosis was the predominant type. The visceral form showed a gradual increase in incidence and, coincident with the advent of modern methods of incubation for commercial chicken raising, has become the most prevalent. It also became increasingly apparent that it was far more contagious than the other leukoses and, for this reason, and the fact that it was of economic importance, efforts to control the spread of this disease seemed feasible and received financial support.

Regardless of the motives for conducting studies with this virus, the results have contributed much to the general problem concerning the tumor viruses. The virus of visceral lymphomatosis is a *contagious* tumor virus and its transmission by way of water and excreta dispels the idea that tumor viruses have unique and mysterious routes of transmission which enable them to survive and gain access to new hosts. Even transmission via the egg is not an exclusive property of this virus because other disease-provoking agents (Fuller, 1956) are known to be transferred in like manner. It does, however,

indicate that this tumor virus has solved the problem of transmission from generation to generation and this gives it better assurance for survival. Of the known tumor viruses, none has yielded easily to the epidemiological approach aimed at prevention of the disease.

Efforts to elucidate the contagious nature of this virus have contributed another generalization which is of importance to those interested in the control of cancer. As the work progressed it became clear that the exposure of a virus as the causative agent of this tumor was no assurance that the disease would yield readily to preventive measures. Discovery that the virus was egg-borne showed why the usual procedures for the prevention of contact infection were almost hopeless, and suggested as control measures the acquisition of stocks of chickens which were highly resistant to the virus, or the almost impractical procedure of maintaining chickens in virus-free quarters. Indeed, it now appears that the more practical approach to control resides in the discoveries of antibodies in eggs and susceptibility of newly hatched chicks. These leads can be exploited by immunizing the hen before the eggs are laid, in hopes that the chicks will be protected from infection during the brief and critical period after they are hatched.

Work with the virus of visceral lymphomatosis has made another contribution to the basic knowledge of tumor viruses. This stems from its established relationship to other viruses of the fowl leukosis complex. All who review this interesting problem emphasize the similarities and dissimilarities of various diseases of the complex and of the viral agents implicated in their causations. It is the consensus that while each disease and virus possess distinct properties, all the diseases, as well as all the viruses, possess undeniable relationships. Another review of the investigation leading to this generalization is not essential to the present discussion, for this was done in an excellent summation by Oberling and Guérin (1954). Instead, a brief account of the recent publications dealing with the subject will serve to illustrate several avenues of approach and the attending results.

Beard's (1957) review of the contributions from his laboratory deals for the most part with two diseases of the complex: erythroblastosis, characterized by the presence of large numbers of erythroblasts, and myeloblastosis, by large numbers of myeloblasts, in the circulation. The viruses of both diseases were found in blood plasma and, with this as the point of departure, a series of investigations contributed significantly to knowledge of the biological, physical, and chemical properties of the viruses. The blood plasma from individual chickens showed a wide range in the number of virus particles and members of the most susceptible stock of chickens varied widely in their degree of susceptibility to the two viruses. Fewer erythroblastosis than myeloblastosis particles were needed to induce disease and the erythroblastosis virus elicited symptoms and killed its hosts earlier. Three-day-old

chicks were most susceptible to the virus of myeloblastosis and resistance increased with age, whereas the converse is true for the erythroblastosis virus, which is far more lethal for 77-day-old than for 3-day-old chickens. Chemical studies revealed that the virus of myeloblastosis showed a pronounced activity to dephosphorylate adenosine triphosphate but the virus of erythroblastosis was lacking in such activity.

Serological studies, however, in common with earlier efforts, suggested a strong relationship between the viruses. Sera from chickens actively immunized against either virus neutralized both viruses. Of interest was the finding that rabbits injected with myeloblastosis virus or normal chicken tissues developed neutralizing antibodies for both viruses. These results were interpreted as evidence of similar antigens in the viruses. A difference in antigen components was suggested by the observation that the administration of guinea pig kidney tissue to rabbits produced antibodies capable of neutralizing the myeloblastosis virus but incapable of neutralizing the virus of erythroblastosis. Beard interpreted these results as suggesting: (1) a similar antigenic component in the viruses; (2) a second antigenic component intrinsic to both viruses that was derived from, or associated with, normal chicken tissues; (3) a third component, presumably the Forssman antigen, associated with the myeloblastosis virus but not with the erythroblastosis virus. Thus, despite biological and enzymatic differences, serological tests indicate a close relationship between the two viruses.

Burmester (1957) observed a difference between the neural and visceral forms of lymphomatosis. During the course of investigations in which the egg-borne transfer of visceral lymphomatosis was established, he noted, "only visceral lymphomatosis occurred to any significant extent in the test chicks inoculated." This finding, together with other negative data on egg transmission of the neural form, was interpreted by Burmester as indicating different causative agents for the two diseases. Apparently, cross-neutralization experiments between sera from fowls actively immunized against the neural, and visceral forms have not been performed, but Beard (1957) reported that the sera of chickens immunized against the myeloblastosis virus contained neutralizing antibodies for the virus of visceral lymphomatosis.

This brings us to the point where the fowl tumor complex can be enlarged to include not only the leukoses but other virus-induced connective tissue tumors of fowls. Oberling and Guérin (1954), as well as Duran-Reynals (1953), have reviewed and discussed adequately the evidence for the concept of a group of related viruses acting as causative agents for a variety of fowl tumors. Serological studies revealed natural antibodies to a number of sarcomas in adult chickens, in chicks, and in the egg yolk, while cross-neutralization tests between sera from actively immunized chickens or pheasants revealed definite relationships between the viruses. These findings

naturally led to the conclusion that the tumors were induced by a group, or family, of related agents. During their discussion of these serological studies Oberling and Guérin (1954) insert the following pertinent statements: "The fowl is one of the least suitable animals for serological researches. . . . Perhaps there are some natural antigens related to the sarcoma viruses which the fowl absorbs and thereby immunizes itself. . . . It is essential to avoid regarding the appearance of the natural antibodies as indicating some relationship between these viruses and the normal constituents of the cells that they may infect. . . . It thus seems clear that the viruses of the avian sarcomas contain two types of antigens, one being virus proper, the other of fowl origin."

Despite their timely reminders of pitfalls to avoid in the interpretation of serological work, Oberling and Guérin (1933) presented other data which supported the idea of interrelationships in fowl tumor viruses, namely, the results attending the introduction of the viruses into susceptible hosts. They were the first to show that the inoculation of fowls with tissues from leukemic organs or leukemic blood produced sarcomas and that these sarcomas, upon passage to other fowls, produced leukemia. In some animals tumors arose at sites remote from the inoculated tissue; these included a variety of tumor types. Such observations present one of the most interesting problems encountered with the tumor viruses. Are the different types of fowl tumors dependent upon infection of cells by viruses specific for certain cells, or can a single virus enter different cell types and provoke them to neoplastic proliferation? And, when added to the possiblity of a single virus producing a variety of tumors is added the well-known genetic lability of viruses and their widespread distribution in nature, the potential for discovering tumor viruses becomes almost limitless.

Reasoning along these lines is perhaps why some investigators are inclined to regard the virus of visceral lymphomatosis, because it is contagious, as the stem virus for others included in the fowl tumor complex (Beard, 1957). Regardless of whether the agent of visceral lymphomatosis is the stem virus for a family of tumor viruses capable of inducing tumors in fowls, the idea of a group relationship between fowl tumor viruses and of one virus eliciting a variety of tumor types may some day be looked upon as an important basic concept. Extension of the idea of groups of tumor viruses having a common kinship in a single ancestral or stem virus leads directly to the possibility that any virus capable of exciting cell proliferation could, under suitable environmental conditions, act as a tumor virus.

Thus, the rewarding efforts with the fowl tumor viruses, especially those revealing the routes of transmission of the visceral lymphomatosis virus and those suggesting a related group of viruses, lead straight to some major problems concerning the tumor viruses, namely, further elucidation of the group

and unitarian concepts of virus relationships and activities. While the attack on these problems with established viruses has produced gratifying results, it may be more advantageous to explore them through the use of viruses recently recovered from the natural disease. Such viruses would not be remote relatives as must be those maintained in the laboratory for many years. The virus of visceral lymphomatosis may be suitable for this purpose and a thorough study of the lesions through the techniques of tissue culture may throw considerable light on the problem.

Before closing this discussion of the virus of chicken visceral lymphomatosis, it is essential to recall once again the many unsuccessful attempts to expose viruses in both spontaneous and induced tumors of fowls (Peacock, 1957). The inability to establish viruses as the causative agents of all tumors of like histological structure is not limited to fowl tumors and will be mentioned later in this chapter in connection with mammary cancer of mice. Hence, there is no need for further discussion at this time, except to state that viruses may not be responsible for the occurrence of all tumors and, to quote from Rous (1936), "the future can be left to take care of it."

B. Rous Sarcoma of Chickens

The discovery of a virus as the etiological agent of a chicken sarcoma by Rous (1910, 1911) was a basic observation which added much to knowledge of tumor viruses. More than forty-five years later, this virus continues to be one of major interest to cancer workers, to confront them with some of the most difficult and interesting problems, and to give them encouragement in their efforts to search for a similar agent in other neoplasms. The immediate result of the observation was the stimulation to search for viruses in other tumors of chickens; this search uncovered viral agents in a variety of fowl sarcomas (Rous, 1936). The virus of Rous sarcoma, however, has remained the agent of choice throughout the intervening years because it has the advantage of always inducing the neoplastic state at the site of application and does so in a matter of days. For these reasons, it is the most satisfactory of all tumor viruses in studies designed to explore the relationship the virus bears to its animal host and the cells it infects.

Recent reviews of the Rous sarcoma virus by Harris (1953), Bryan (1955), and Bryan *et al.* (1955) included all pertinent references to previous investigations and summarized the progress achieved. Hence, this discussion will concern only those facets of the general problem which are now receiving most attention, and, will attempt to interpret the implication of recent work. It is hoped that the best procedure will be a consideration of the problems of this tumor virus, which include: (1) host-virus relationships and, (2) biological properties of the virus.

1. *Host-Virus Relationships*

Many factors are known to be involved in the interactions between the Rous sarcoma virus and its hosts but, in recent years, the age factor has probably received most attention. Duran-Reynals (1953) has emphasized the importance of age in the response of the host to a number of tumor viruses and has used the virus of Rous sarcoma extensively in his investigations. He (Duran-Reynals, 1940a) observed that administration of the virus to chicks produced hemorrhagic areas around small blood vessels because non-neoplastic lesions developed in the vessels and these lesions contained abundant virus. With advancing age, chickens became more resistant to the virus which, instead of provoking "destructive" lesions, induced cell proliferation that proceeded to malignancy. Likewise, young animals responded to small amounts of virus by developing rapidly growing tumors which often metastasized, whereas adults required larger quantities of virus before developing tumors that showed less tendency to metastasize. These reactions between host and virus are consistent with those of other virus-induced diseases when age renders the older animals more resistant, but Duran-Reynals considered the age of the host as being of paramount importance in the successful adaptation of the Rous sarcoma virus to other species.

He found it was impossible to obtain permanent adaptation of the virus when it was transferred from chicks to very young ducks, but adaptation did occur when day-old ducks received the virus from tumor grown in 3- to 10-month-old chickens. Thus, the age of the inoculated duck and the age of the host chicken supplying the virus were the important factors in successful adaptation of the virus to a heterologous host. Duran-Reynals interpreted the age factor as playing a far more important role than rendering the young more susceptible, in that the virus, while residing in the older and more resistant chicken, underwent alterations which increased its capacity to infect a foreign host. This concept of the influence of the host's age upon the infectivity of tumor viruses presents a challenging problem to the cancer investigator because if it can be applied successfully to other tumor viruses, especially to those not previously exposed to many passages in the laboratory, it could offer an explanation for the induction of different types of cancer by a single virus.

It has been known for years that the age of the host is important in its response to virus infections and all reviews of the tumor viruses emphasize the importance of the age factor, particularly in relation to successful efforts at heteroinoculation of the Rous virus (Andervont, 1957). While the variability and adaptability of the virus must be of importance in its ability to evoke responses in its hosts, some of the most recent work suggests strongly that such responses can be conditioned by the quantity of virus used as inocula. Bryan (1957), in a recent summation of the host-virus relationships of

the tumor viruses, stated: "The virus-tumor problem is, therefore characterized by a diversity of specific patterns of interplay between virus quantity and level of host susceptibility, in determining the nature and outcome of the virus-host interaction leading to neoplasia." Bryan (1957) used the Rous sarcoma virus in a series of brilliant experiments on the reactions between it and its host and showed that the incidence of tumors, the latent period, the rate of tumor growth, the tumor type, the amount of recoverable virus in the tumor, and the survival of tumor-bearing chickens were all correlated with the amount of virus used as inoculum and concluded: "this particular virus is the direct activating cause of the cancerous reaction which it elicits."

Bryan's contributions are of basic importance to the problems of the tumor viruses, not only because they established a correlation between the biological properties of the Rous sarcoma and the amount of etiological agent used to induce it, but they exposed a fundamental difference between the Rous virus and ordinary viruses. The Rous virus, once it takes residence in a cell, increases slowly and provokes the cell to proliferation, while the ordinary viruses replicate rapidly, destroy the cell, and escape to enter other cells. This difference in rate and manner of increase leads to the presence of many infectious particles of ordinary viruses in intercellular spaces and explains how humoral antibodies can limit the course of many virus infections, but have little influence upon an established Rous sarcoma.

Another significant contribution from these studies (Bryan *et al.*, 1955) was the discovery that tumors produced with small amounts of virus yielded little or no extractable virus. Such findings with a known highly infectious tumor virus show that a virus can cause a tumor and remain undetectable.

Groupé and Rauscher (1957b) have extended observations with these "nonviral" tumors to growths induced by the Rous virus in a heterologous host. Turkeys, 3 to 6 days of age, were as susceptible as chicks to the tumor-inducing activity of the virus, but the virus was detectable only in those turkey tumors produced with large quantities of virus. Further, during serial passage in young turkeys, the virus displayed a progressive loss in potency and, in the fourth passage, produced tumors in only 3 of 19 turkeys. An extract of these 3 tumors did not contain demonstrable virus and failed to produce a tumor in 25 turkeys. These results could only be attributed to the quantity of Rous virus instead of to a qualitative change in the virus which enabled it to evoke tumours in a foreign species.

In other investigations, Groupé *et al.* (1957b) carried the Rous virus through 35 serial intracerebral passages in newly hatched chicks. Brains used for passage material were taken from chicks succumbing earliest to infection and bioassays were performed to determine the relative potencies of the brain passage materials. After the first 17 intracerebral passages there was a progressive increase in potency of the brain-passage virus and, beginning with

the twenty-second passage, there was increasing frequency of "typical hemorrhagic disease" in the inoculated chicks. Hepatic lesions appeared during the twenty-fifth passage and also occurred with increased frequency in subsequent passages. Tests for correlation between the dose of virus and incidence of hemorrhagic disease and hepatic lesions showed that both were associated with the inoculation of larger amounts of virus, but more virus was required to provoke liver lesions than the hemorrhagic disease. Finally, a large dose of the virus used as source material for the brain passages also produced both hemorrhagic disease and liver lesions in 1-day-old chicks. Thus, the occurrence of liver lesions and hemorrhagic disease was dependent upon the quantity instead of the quality of the virus.

Experiments of this sort do not refute the idea that tumor viruses are subject to alterations of a genetic nature, but they do serve as a warning signal to those who are prone to interpret a change in the response to a virus as a change in the virus itself without first excluding a quantitative relationship between host and virus or a qualitiative change in the host.

Various substances have been tested for their abilities to alter the response of the host to the Rous sarcoma virus (Harris, 1953). A recent publication by Groupé *et al.* (1956a) serves to illustrate how treatment of the host can influence its reaction. These investigators found that the administration of hydrocortisone to chicks less than 7 days of age gave different results, depending upon the dose of hydrocortisone and the time of virus administration. When daily large doses of hydrocortisone were started 2 days before inoculation, the tumors appeared later in treated than in untreated control birds; when small doses were used, tumors occurred in experimental and control animals at the same time, but the experimental animals developed larger tumors. Further, the tumors in animals receiving larger amounts of the compound were firm and sharply circumscribed, in contrast to the soft and invasive tumors in the controls. It is of special interest that soon after cortisone treatment was stopped the altered tumors grew rapidly and soon resembled those of the control animals. When daily large doses of hydrocortisone were started 2 days after the virus was administered, the tumors were invasive and grew more rapidly than those in the control animals. The timing and dose of the compound exerted a pronounced influence upon the host's reaction to the virus.

Groupé *et al.* (1956b) reported the results of daily injections of Xerosin started 2 days before inoculation of 2 to 5-day-old chicks with varying amounts of the Rous virus. Xerosin delayed the occurrence of tumors in the treated chicks but was most effective in chicks that had received small amounts of virus; that is, the effectiveness of Xerosin was determined largely by the amount of virus used to initiate the tumor. Injections of Xerosin also produced firm and circumscribed tumors similar to those appearing after treatment with

large doses of hydrocortisone but, in contrast to the latter tumors, those altered by Xerosin did not revert to the typical tumor found in control birds. These investigations, in which young chickens were used, showed that extraneous factors affect the host-virus relationship.

Cortisone and X-irradiation, alone or in combination, are known to lower the resistance of animals to the growth of homologous or heterologous tumor cells, and cortisone alters the susceptibility of animals to virus infections (Andervont, 1957). The use of such measures should be explored with virus-induced tumors to ascertain whether, as with the Rous virus, the viruses change their affinities for tissues and species. The inhibition of growth of the Rous sarcoma in young chickens by the administration of Xerosin or hydrocortisone could be used to test the suggestion of Duran-Reynals that the Rous virus while under adverse conditions, is more conducive to change which enables it to produce tumors in heterologous hosts. Cortisone could also be used in attempts to lower the susceptibility of adults of a foreign species to the Rous virus to ascertain whether they would then accept the virus as readily as the more tolerant young animals.

One handicap to early work with the Rous virus was the lack of genetically homogeneous test animals. Perhaps one of the most serious questions facing today's investigators is the advisability of a re-evaluation of factors affecting the virus-host interactions through the use of chickens of known genetic constitutions. In view of the proved usefulness of inbred animals in cancer research, it may be assumed they would be of considerable assistance to investigators working with the Rous virus. As one reads the case for the influence of age upon the activity of the Rous virus, it suggests to those who have used inbred animals that the effects attributed to age could also reflect the susceptibility of the host. Also, the summation of serological studies with the fowl tumors by Oberling and Guérin (1954) suggests that many of the disconcerting results could be clarified through the use of inbred animals. The best approach to the separation of genetic and other factors in host-virus interactions is through the use of animals of known genetic make-up.

Inbred lines of chickens exhibiting differences in susceptibility to the Rous virus have been developed by Waters (1945, 1951) and by Greenwood *et al.* (1948). Bryan (1957) reported preliminary findings with two lines established by Waters. The average effective dose of Rous virus was 10,000 times larger for the resistant than for the susceptible line. Availability of such animals enables the investigator to ascertain the response of animals of different genetic constitutions to a standard amount of virus or vice versa. Information of this sort may be of much assistance in studies of a tumor virus, which is almost ideal for quantitative investigations because of its ability to elicit the change to malignancy within a matter of days.

According to Harris (1953), Carr (1942) injected the Rous virus into a relatively nonsusceptible line of fowls and observed regression of tumors, but as long as a year later tumors occurred at the injection site. Bryan (1957) reported the occurrence of tumors resembling Rous sarcomas at sites remote from the site of application as long as 90 days after the chickens had received the virus. The ability of this infectious tumor virus to persist, presumably in cells, for long periods of time without provoking overt tumors suggests that unknown factors exert some control over the cancer process. The use of inbred hosts in studies of host-virus relationships would assist in the identification of these factors.

2. *Biological Properties of the Virus*

It is impossible to draw a clear distinction between the properties of the Rous virus and the reactions between it and its host, but, for the sake of convenience, such properties as variation and adaptation, masking, multiplication and assay, as well as the problems of purification, are best discussed in relation to the virus itself.

a. Variation and Adaptation. Variation and adaptation of the virus have been discussed adequately by Harris (1953), Oberling and Guérin (1954), and Dmochowski (1957). Here, such changes in the Rous virus are considered as they occur in the chicken, its natural host, and in other species, its heterologous hosts.

Variation, or deviation from the normal sequence of events within the natural host, is a common trait in viruses, and such variation usually occurs when the virus gains access to a new type of host cell, acquires a greater degree of activity, or is introduced into a host of high susceptibility. It would be surprising if the Rous virus, during the many years of animal passages in laboratories throughout the world, had not shown variations in activity. Older workers will recall the common practice, now forgotten, of adding a trace of kaolin or kieselguhr to a filtrate to facilitate virus activity, and the difficulties of performing an intravenous injection without producing a subcutaneous tumor at the site of needle puncture. In a recent paper, Groupé and Rauscher (1957a) described the growth of the virus in chick brain and, while the presence of Rous sarcoma cells beneath the meninges could have accounted for an increase in virus, there were excellent reasons for assuming that the virus was propagated in the brain tissue. In either event, the authors concluded: "the growth curve of Rous sarcoma virus in chick brain paralleled that of many non-neoplastic animal viruses." It will be recalled that the same investigators with Bryan (1957) found that further passages in chick brains produced a highly active virus which, when introduced intracerebrally, produced typical "hemorrhagic disease." As stated

previously, such observations suggest that variations of the virus within its natural host can be the result of either quantitative or qualitative changes.

Variations of the virus in heterologous hosts have received considerable attention and have proved interesting. Duran-Reynals (1953) performed the pioneer work in this field and obtained best evidence of virus variation when the duck was used as the heterologous host. The virus was administered to day-old ducklings and they developed two types of lesions. The first arose within 30 days, could not be propagated in ducks but, upon reinoculation into chickens, produced the typical Rous sarcoma. The second appeared several months after inoculation, could not be transferred directly back to chickens, but produced a new tumor type in ducks. Duran-Reynals interpreted the results as showing that the second type of duck lesion was, in reality, induced by a variant of the Rous virus. Some duck variants could, by suitable procedures, be reversed to produce tumours in chickens and some of these tumors differed from the original Rous sarcoma.

This brief account does not include all the interesting observations accompanying heteroinoculation of the Rous sarcoma virus, but it is hoped that sufficient information is presented to show that this virus may, with suitable experimental procedures, produce tumors in foreign hosts. Duran-Reynals used large amounts of inocula in his efforts to infect other species and, although no experimental evidence is available, it is reasonably safe to assume that large amounts of virus were introduced. It would be of interest to know whether the highly potent material procured by Groupé *et al.* (1957b) is capable of infecting adult ducks.

When the interesting work with variants of the Rous virus is considered in retrospect, it is clear that this virus possesses properties similar to those of other viruses. Fluid from an innocuous fever blister, when introduced into the corneal epithelium of the rabbit, can initiate a reaction which terminates in a fatal encephalitis. Newly hatched chicks are more susceptible than full-grown chickens to cutaneous, corneal, or intracranial inoculation with the virus of vaccinia, and suckling mice are susceptible to Coxsackie viruses when adults are almost completely resistant (Andervont, 1957). By this time, the reader may be aware that the purpose of this chapter is to show that the tumor viruses, in common with other viruses, display a wide range of biological properties and that the major problem concerning the tumor viruses is not to reveal wherein they resemble or differ from other viruses but to emphasize the importance of ascertaining why the tumor viruses incite unlimited cell proliferation and how many tumors are caused by viruses.

The remaining properties of the Rous virus have a direct bearing upon the relationship between it and the cell and are, therefore, more closely concerned with the tumor process.

b. Masking. The "masking" of tumor viruses is, at the moment, very controversial. The idea that a tumor virus can reside in, or combine with, the host cell in such a manner that it is not detectable by known techniques has been advanced for many years. Indeed, no reference is given in support of the statement because it could be a separate problem of the tumor viruses to give priority to the first investigator who expressed the idea in writing. Anyone familiar with virologists interested in the problems of cancer has heard this and other equally interesting theories expressed on numerous occasions. The term "masking" has been challenged recently (Beard, 1956) on the basis that the phenomenon is dependent upon the presence of a small number of virus particles and not upon a difference in the state of the virus. The controversy may be a matter of semantics. Perhaps a virus responsible for the naturally occurring disease should be used to test the idea instead of a "laboratory trained" virus, or possibly the electron microscope may not differentiate between living and dead virus particles. Regardless of the outcome of the controversy, a number of investigators consider seriously the possibility that a virus can enter into a cell, combine with cellular components, and thereby stimulate cell proliferation but not undergo replication to produce new and infectious virus particles. With this understanding of the term "masked" the investigator is faced with the problem of learning either how to expose the hidden virus or to develop reliable techniques for the detection of cells altered by the virus.

It has been known for years that, at times, filtrates prepared from Rous sarcomas do not contain virus activity. During such periods, the implantation of living tumor cells is necessary for transmission of the tumor to new hosts (Gye and Andrewes, 1926) and this has been interpreted (Duran-Reynals, 1953) as the effect of a temporary masking of the virus. Bryan *et al.* (1955), in the discussion of their work with the initiating dose of Rous virus, review the earlier observations and suggest that small quantities of virus in the inocula, together with the use of resistant chickens, could account for the absence of detectable virus in the filtrates. Again we are confronted with the lack of quantitative studies in early investigations with this virus, and perhaps the use of resistant chickens, to supply an answer to the question whether the highly infectious Rous virus can become masked for any appreciable period of time. Indeed, one of the basic problems of the Rous sarcoma virus is to determine whether, in contrast to the tumors induced with chemical carcinogens, the proliferative power of the cells of this tumor is dependent upon the presence of the virus in them. Calnan *et al.* (1957) attacked this problem through the use of a tumor initiated with a minimum effective dose of virus (Bryan *et al.* (1955) and on the basis of experiments showing that the rate of growth of Rous sarcomas was associated with the amount of virus they contained. From the original tumor three lines of transplantable tumors

were established. The first, designated the "stem line tumor" arose directly from the original tumor. It was carried through 25 serial passages in chickens and at each passage the slowest growing tumor which did not deviate markedly from the other tumors supplied tissue for the next passage. The second line was started from the eighteenth passage generation of the stem line tumor in which a chicken developed an aberrant, slowly growing tumor. This line represented a deliberate effort to establish a virus-free tumor; older chickens were used and the slowest growing tumor of each passage was used to inoculate chickens for the next transfer generation. This line was carried through 13 serial transfers before it was terminated because it failed to grow in the next passage. The third line was started from the eighteenth generation of the stem line in which a chicken developed a relatively fast-growing tumor. Subsequent passages were made from the fastest growing tumor of each transfer generation.

Each tumor used for passage was assayed for the virus. Extracts of the rapidly growing third line tumors contained relatively large amounts of virus. Extracts of tumors from the first and second lines showed wide variations in virus content but neither line became free of virus. Bryan (1957) has found that all experimental evidence indicates this virus is intimately associated with the biological characteristics of the tumor cells. This, together with the possibility that the Rous virus is a highly infectious and fixed variant of a stem virus, makes improbable its reversion to the masked state.

c. Multiplication and Assay. Studies of the multiplication or replication of the Rous virus, as with similar studies of other viruses, include not only the increase in virus quantity but also the rate of virus production by infected cells, the quantitative correlation between detectable virus particles and virus activity, and the use of assay procedures to correlate the number of virus particles with increased activity. The chief objective of such investigations is to ascertain the mechanisms of virus carcinogenesis.

Sanford *et al.* (1952) used tissue culture techniques to ascertain the cell type capable of supporting multiplication of the Rous virus. The efforts of previous investigators were summarized in detail, beginning with Carrel and Ebeling (1926), who concluded that the monocyte and not the fibroblast was the infected cell, and including the findings of Ludford (1937) and those of Halberstaedter *et al.* (1941). In both latter studies the conclusions were contrary to those of Carrel in that the fibroblast was the infected cell.

Sanford and her colleagues conducted a thorough study of the problem by exposing cultures of chicken fibroblasts or monocytes to filtrates containing the Rous virus and, after varying intervals of time, testing the cultures for virus by injecting them into chickens. The virus remained active at 37.5°C. for 4 days in a mixture of horse serum, balanced saline, and dilute chick embryo extract. In cultures of monocytes the virus survived for 11 to 14 days

but did not remain active beyond this period, whereas in cultures of fibroblasts virus activity continued for 6½ months, at which time the experiment was terminated. They concluded that the fibroblast is probably the host cell of the virus in the tumor.

Lo *et al.* (1955) found that, in tissue cultures of normal chicken fibroblasts grown in a clot substrate and infected with the Rous virus, the cells became altered within 8 to 14 days. These cytopathogenic effects consisted of degenerating cells and lysis of the clot. Serial subcultures from these degenerating cultures grew vigorously and appeared healthy for a time, but then hypertrophied cells were produced, of which most disintegrated but some developed into giant cells. All infected cultures were characterized by a variety of cell shapes and sizes, while cells in the noninfected control cultures remained uniform. They also stated: "The morphological alterations in the infected chicken fibroblast cultures were identical with those seen in cultures of Rous sarcoma tissue."

In view of the recent interest displayed by virologists in tissue culture, it is strange that more have not used it to culture or assay the Rous virus. Manaker and Groupé (1956) showed the possibilities of this approach when they observed discrete foci of proliferative activity in tissue cultures inoculated with the virus; the number of foci was directly related to the amount of virus in the inoculum. Such findings promise a rapid method for quantitative assay of virus suspensions.

Considerable work is in progress to procure rapid methods for assaying the Rous virus. One promising procedure is based upon the observation by Rous and Murphy (1913) that the virus is capable of inducing hemorrhagic lesions in chickens. This "hemorrhagic disease" was studied intensively by Duran-Reynals (1940a), who found a higher incidence in very young animals, while Milford and Duran-Reynals (1943) showed that chick embryos, when inoculated intravenously, developed this lesion instead of tumors.

Lo and Bang (1955a,b) made a careful study of the factors involved in the occurrence of the lesions in embryos and found their production was favoured by the intravenous route of inoculation, the age of the embryo, and the temperature of incubation. With this information at hand, they developed a method for titrating the virus which was suitable for the determination of relative potencies of virus preparations and for measuring neutralizing antibody against the virus. Borsos and Bang (1957) reported a quantitative relationship between the number of lesions and the amount of virus used as inoculum.

Development of another assay procedure had its roots in the discovery by Rous and Murphy (1911) that Rous sarcoma cells, or filtrates, produced tumors on the chorioallantoic membrane of the developing chick embryo. Keogh (1938) designed a technique for procuring discrete ectodermal or mixed

mesodermal and ectodermal lesions in the membrane, and established the feasibility of using the discrete lesions for virus titration. Groupé *et al.* (1957a), with a refined procedure, procured a correlation between the number of lesions and the quantity of virus used to infect the membrane.

Prince (1958a) recognized that the variability between embryos in their responses to infection with the Rous virus was a major deterrent to the use of Keogh's technique for quantitative assays of the virus, and has done much to make it practical. He tested embryos from 9 stocks of chickens for susceptibility to the virus and found a wide variation in the responses of their membranes. Embryos from one inbred line of chickens were highly susceptible and proved to be satisfactory for quantitative assay. Prince (1958b) then used the most susceptible and most resistant lines of chickens to ascertain the nature of the susceptibility to the virus. Sera from adult chickens were tested for neutralizing antibodies to the virus; it was found that sera from chickens whose embryos were most susceptible contained the most antibody. This led to a test for antibodies in membranes from the susceptible embryos because about 10 % of these were resistant to the virus: membranes from susceptible and resistant embryos showed no essential differences in their neutralizing activities. These results indicated that humoral antibodies were not responsible for the resistance of the membranes. When embryos procured by reciprocal matings between susceptible and resistant chickens were tested with the virus, their membranes proved to be equally susceptible. It was concluded that genetic factors, and not humoral antibodies, were responsible for the resistance of the chorioallantoic membrane to the virus.

Rubin (1957) applied the technique in conjunction with tissue cultures and other methods to perform a series of interesting studies on cell-virus relationships. He confirmed Keogh's findings that the number of lesions produced upon the chorioallantoic membrane was dependent upon the concentration of virus in the inoculum, and he interpreted this as suggesting that each tumor on the membrane was caused by a single virus particle. Tissue cultures of Rous sarcoma cells were assayed for the virus by exposing membranes to the supernatant fluids. By means of this procedure it was determined that the rate of virus production per cell was low but remained constant for a considerable time because of the intimate relationship between cell and virus. This indicated that, in contrast to bacterial viruses, the Rous sarcoma virus was produced slowly by most sarcoma cells but these cells produced virus for a long time. Through determinations of the amounts of intracellular and extracellular virus, Rubin concluded that the average infectious virus particle stayed within the cell for one-half hour after its completion. Also, the rate of virus production correlated with an increase in cell size and, because this increase took place only in the cytoplasm of the cell, it was inferred that virus formation took place within the cytoplasm.

Pontén's (1956) investigations are pertinent to the rate of Rous virus production. This investigator did not use tissue cultures but found that ascites tumors of Rous sarcoma origin showed virus activity in direct proportion to the growth rate of tumor cells as well as to the elapsed time.

Epstein (1956), working along the same lines, found a correlation between the biological activity of the Rous virus and the number of cells containing visible virus particles. His main objective was to ascertain whether biological assay would support the detection of virus particles by means of the electron microscope, because such controls were lacking in previous studies by electron microscopists. Examination of the cells from 13 ascites tumors derived from the Rous sarcoma revealed that the incidence of cells containing visible virus particles ranged between 1 in 50 and 1 in 3000 in 9 tumors, with no detectable particles in 4 tumors. The particles were located in, or in the walls of, cytoplasmic vacuoles of tumor cells, with an average of 100 particles per cell. He tested 5 tumors for virus activity and obtained a correlation between the incidence of cells containing particles and the tumor-producing ability of tumor extracts. This permitted the conclusion that the particles were visible forms of the Rous virus.

While, at first glance, Epstein's observation that only 168 of 27,637 tumor cells contained particles could be interpreted as not confirming the work of Rubin, there is, as pointed out by Epstein, no disagreement if only a small proportion of tumor cells contained infectious virus particles, whereas the remainder contained a nondetectable stage in the development of the virus. This interpretation is also in harmony with the suggestion of Carr (1947), who calculated that not all the virus in a Rous sarcoma could be infectious.

Haguenau *et al.* (1958) approached the problem of cell-virus relationships by fulfilling three criteria they considered essential: (1) the inoculation of cell-free material of known potency to induce the tumors, (2) dilutions of the cell-free material to establish quantitative relationships between the inocula and resultant tumors, (3) determinations of the activities of extracts from the resultant tumors. With these criteria in mind, they induced tumors with variable amounts of virus, prepared thin sections of the tumors and examined them with the electron microscope, and performed bio-assays of the induced tumors. In addition, electron micrographs were made of pellets obtained from virus suspensions of the induced tumors. The results of the combined biological and electron microscopic studies revealed statistically significant differences between tumors evoked with large and small amounts of virus in (1) the number of cells containing particles, (2) the number of particles per cell. Within the tumors induced with large concentrations of virus, there was a correlation between the number of particles and the activity of the tumor extracts. Pellets from tumors induced with large amounts of virus contained many more particles than those induced with small amounts.

The authors interpreted these results as indicating that the particles represented, or were quantitatively related to, the Rous virus. The high incidences (70–95 %) of cells containing particles in tumors induced with cell-free materials of high potencies suggested that the detectable particles were responsible for the tumors, thereby obviating the necessity of postulating a nondetectable stage in the development of the virus. Further studies with closer serial dilutions of virus are needed to settle this point.

d. Purification. The problem of purification of the Rous virus has proved to be extremely difficult, but the by-products of a sustained effort by Bryan and his colleagues to attain this goal have been so rewarding that the final achievement will have long since contributed much to the major problems of the tumor viruses.

A recent review by Bryan and Moloney (1957) of the purification problem makes unnecessary another summation. Many obstacles have been overcome. Use of citrate buffers (Bryan *et al.*, 1950; Bryan, 1955) aided much in stabilizing this extremely fragile virus and present studies are aimed at methods for the prevention of harmful water-soluble products of fatty-acid oxidation. Progress has also been made on the problem of dissociating the virus from the large quantity of nonviral material in tumor extracts.

One major problem in purification of the virus is to procure starting material with a sufficiently high virus content. This appears to have been essential for the purification of other tumor viruses (Beard *et al.*, 1955) and is, in all probability, the next major problem in the purification of the Rous virus. The time-honored procedure of selecting "tumors of especially rapid growth," as described by Rous (1911), together with the use of the most potent virus suspension for each animal passage, may supply an answer to this problem. Bryan and Moloney (1957) reported some success with this technique and also suggested the use of tissue cultures. As stated previously, it is strange that the Rous virus has escaped notice by those interested in the propagation of viruses in tissue cultures.

This summation of recent work with the virus of Rous sarcoma is presented to illustrate how this virus is now being used to elucidate cell-virus relationships which must be of importance in the occurrence of this virus-induced tumor. These efforts are, therefore, an extension of the chief contributions the Rous virus has made to the tumor virus problems. It has always served as the "pilot" virus for other tumor viruses and is now used to compare a tumor virus with other viruses as soon as any advance is made in studies with plant, bacterial, or animal diseases. It should always be kept in mind, however, that results with one tumor virus may not apply to others. And this could apply to the Rous virus because many years of serial passage through large numbers of hosts could have done much to alter its biological properties. Rous (1910) found that successful propagation of the original tumor cells was possible

only in "the small, intimately related stock in which the growth occurred" and, apparently, the virus was not detected until the sixth transplant generation through susceptible hosts. One wonders whether tissue cultures of the primary tumor would have exposed the agent.

Before leaving the fowl tumor viruses, it is fitting to acknowledge that investigations with these viruses have built a firm foundation for almost all other work with tumor viruses and, after years of investigation, the two used here as prototype viruses are probably the most useful in present-day research. The Rous virus continues to be the most valuable for studies on cell-virus relationships, while the virus of visceral lymphomatosis, because it is available in the natural state, continues to be a test virus for the evaluation of concepts concerning the origin, spread, and biological relationships between tumor viruses.

C. Papillomatosis of Rabbits

Shope (1933) discovered that a virus was implicated in the origin of cutaneous papillomas found in cottontail rabbits living in certain regions of the United States. The benign lesions produced with the virus would probably be of minor interest to oncologists if they had remained papillomatous, but the discovery by Rous and Beard (1935) that they changed to malignant epidermoid carcinomas focused attention upon them, and subsequent investigations brought to light one of the most interesting and perplexing problems of the tumor viruses. Adequate reviews of the early work with this virus have been prepared by Rous (1936, 1943, 1946) and of later studies by Ginder (1952), Syverton (1952), and Oberling and Guérin (1951). Hence, this discussion includes few references to older work and deals with (1) transmission of the virus and, (2) the papilloma-to-carcinoma sequence.

1. *Transmission of the Virus*

The disease is characterized by the occurrence of epithelial proliferations of variable size and hornlike structure protruding above and loosely attached to the skin. Syverton (1952) mentions one horn measuring 6 inches in length. Histologically, the disease is limited to the epidermis and consists basically of a multiplication of cells in the lower layers. Apparently the virus is infectious only when brought into contact with the injured basal cell layer of the epidermis. The natural routes of transmission of the virus are not known, but available evidence suggests contact between virus and traumatized epithelium. Dalmat (1958) has shown that, under laboratory conditions, the virus can be transmitted by insect vectors.

The papillomas, in common with those of other species, show a tendency to regression. Syverton (1952) has summarized his observations of regressing

lesions in hosts carrying natural and artificially produced papillomas. Of those occurring naturally in cottontails, 21 % regressed within 6 months and 36 % within the following 18 months; of those induced in cottontails, only 6 % regressed during the first 6 months and none did so thereafter; of those induced in domestic rabbits, 9 % regressed within 6 months and none regressed within the next 6 to 24 months. Thus, the rate in domestic rabbits was similar to that in artificially infected cottontails, but the highest incidence occurred in the naturally infected cottontails. Considerable work has been done to ascertain the reasons for these regressions and the findings were summarized by Ginder (1952). The presence of antibodies in infected rabbits was not responsible because papillomas persisted in animals containing potent neutralizing antibodies in their blood, and the disappearance of the virus from the papillomas was ruled out when highly active virus was found in receding papillomas. Ginder mentioned another finding of interest, namely, that multiple growths on individual rabbits receded at the same time, and concluded: "Some little understood host factor is of great importance." That this factor could be the genetic constitution of the host will be emphasized throughout this discussion.

When the virus was introduced into the traumatized skin of cottontails, papillomas were detectable within 8 or more days and the lesions usually contained the virus in variable quantities, but in some papillomas the virus could not be detected and, on the average, less virus was present in the induced lesions of cottontails than in those of the natural disease. This wide range of virus content between papillomas from different cottontails could reflect the mixed genetic composition of the hosts.

Transfer of the virus from cottontail papillomas to the scarified skin of domestic rabbits resulted in the occurrence of papillomas and disclosed a problem of major importance when it was learned that the lesions often failed to contain detectable virus. This discovery sparked a long series of experiments and a controversy concerning "masked" viruses which is one of the most interesting unresolved problems of the tumor viruses. Syverton (1952), in a discussion of this feature of the disease in domestic rabbits, states: "If cottontail papilloma virus were not available as a reagent to establish immunologic evidence for the presence of virus, the growth would inevitably fall into the category of a nonviral mammalian tumor, even though its viral etiology might be suspect." His statement defines the problem and its implications and indicates the desirability of further study to achieve a solution.

Ginder (1952) has summarized the results of the experimental approach to the problem. The fact that infected domestic rabbits developed antibodies to the virus received much attention and efforts were made to reveal the virus through the application of immunological procedures. Extracts of the

noninfectious papillomas elicited antibodies in rabbits after they had received the material intraperitoneally; this antigenic activity suggested the presence of virus in papillomas, but in amounts too small to produce new papillomas. Next, smaller quantities of virus were exposed in papillomas through the use of procedures which increased the chance of virus survival at the site of inoculation. It was also found that, at times, immune serum could diffuse into the papillomas and combine with the virus. While these experiments showed that small quantities of active virus were present in some induced papillomas, other papillomas failed to yield virus and remained free of antibodies. These latter tumors could have contained insufficient virus to produce tumors when they were assayed by the inoculation technique.

Serial passage of the virus through domestic rabbits supplied indirect evidence of virus survival in them. Shope (1935) was able to carry the virus from a cottontail papilloma through 14 successive passages in domestic rabbits. Selbie and Robinson (1947, 1948) and Selbie *et al.* (1948) also reported 14 passage generations of the virus in domestic rabbits. Of special interest was the finding that, after passages through domestic rabbits, their virus failed to provoke papillomas in cottontails. The inability of the virus to infect cottontails after exposure to domestic rabbit tissues recalls Duran-Reynals' (1953) observation that a variant of the Rous virus, following residence in the duck, failed to produce the typical sarcoma when brought back to its natural chicken host.

Despite these suggestive findings, a variant of the virus which is permanently adapted to the domestic rabbit was not made available. The virus appears to be resistant to modification, a property which is in keeping with its known resistance to chemical and physical agents and its apparent specificity for the epidermis. Perhaps the problem should be attacked through a modification of the host's response to infection. Ginder (1952) describes the "dramatic changes" occurring when chemicals are applied to the skin of the domestic rabbit. Intravenous administration of the virus to rabbits bearing tar-induced papillomas accelerates the growth of the lesions and the application of carcinogenic compounds to the papillomas hastens the change to malignancy. Procedures known to alter the susceptibility of the host to virus infections or to the growth of tumors, such as radiation, administration of cortisone, or the methodology for the production of acquired tolerance, may bring about a change in the cottontail virus which will enable it to grow indefinitely in the skin of the domestic rabbit.

Those familiar with the mammary tumor virus of mice will recognize similarities and dissimilarities between it and the virus of rabbit papillomatosis. The mouse virus will be discussed later, but it is apropos at this time to mention that inbred mice exhibit pronounced variations in susceptibility to it. When strains of mice that do not carry the virus are exposed to it, some

do not develop breast tumors and the virus disappears from them within a few generations; others accept the virus, develop tumours, and transmit it through successive generations. This implies that, as with other virus infections, the host plays an important role in the host-virus relationship. Inbred strains of rabbits are not available at present, but races of rabbits have been established which display striking differences in morphological and physiological characteristics (Sawin, 1955).

Greene has applied techniques of tissue transplantation to a study of the papilloma virus and the results have a direct bearing upon the problem of variant viruses. The skin from rabbit embryos was infected with the virus and placed into the brains of adult rabbits; the virus from embryo skin was then carried through 3 serial passages in domestic rabbits (Greene, 1953a). Papillomas developed in infected embryonic rat skin, following transplantation into the brain or subcutaneous tissues of adult rats (Greene, 1953b). This finding presents an opportunity to ascertain whether the virus becomes modified through residence in a foreign species. In another paper, Greene (1954) included experiments designed to compare the infectiousness of the virus from cottontail and domestic papillomas. Cottontail papillomas, when transplanted into the brain of domestic rabbits or to the subcutaneous tissues of hamsters, retained infectious virus for domestic rabbits, but papillomas from domestic rabbits did not contain infectious virus after exposure to brain tissues of cottontails. Exposure of cottontail papillomas to resistant species (adult rats, mice, and guinea pigs) failed to influence the infectiousness of the virus for domestic rabbits. Greene concluded; "Loss of infectiousness is a function of factors resident in the epidermal cells of the domestic rabbit." In other experiments, Greene (1955a,b) was concerned with problems of immunity and concluded that immunity to the virus was humoral in nature and embryonic rabbit skin retained its susceptibility to the virus after exposure to tissues of naturally resistant hamsters and mice.

These experiments supplied the foundation for a later paper (Greene, 1955c) which included findings pertinent to the present discussion concerned with the adaptability of the virus. Embryonic, newborn, and adult skin from domestic rabbits were exposed to the virus obtained from natural cottontail lesions and then transplanted into the brain of domestic rabbits or the subcutaneous spaces of hamsters. One month later the transplanted tissues were removed and tested for virus by administration to the skin of domestic rabbits. Embryonic skin retained the virus most effectively, and papillomas induced in domestic rabbits with this virus supplied a virus which was carried through 8 serial passages in domestic rabbits. Likewise, when papillomas from cottontail rabbits were implanted directly into hamsters and mice, the virus recovered from the papillomas was found to be infectious through 5 transfer generations in domestic rabbits.

These results were selected from others obtained by Greene because they best serve the purpose of this discussion. Apparently, the virus from lesions of cottontail rabbits was changed sufficiently to produce papillomas through serial transfer in domestic rabbits. Greene mentioned that this "infectious virus" may have differed from the original virus from cottontails because the latter induced multiple and confluent lesions of the skin of domestic rabbits within 2 weeks, whereas the "infectious virus" produced a few discrete lesions and usually required at least 6 weeks to do so. He looked upon the "infectious virus" as a variant form of the original virus, and in his discussion stated: "The infectiousness of Selbie's virus for domestic rabbits was lost after passage in a cottontail, while, in the present instance, the infectiousness of the virus was not altered by such procedure. Despite their apparent differences, the variant viruses possess the significant common property of resistance to the masking effect of adult domestic skin and, as such, offer material for studies of possible pertinence in a clarification of the operation of of the so-called masking process."

Greene's efforts have done more than procure a variant which can be used for the purpose he described. The acquisition of this virus is a very practical achievement, because it established a ready supply of papilloma virus and frees investigators from dependence upon the naturally occurring papillomas which, in the past, have been the only source of virus.

2. *The Papilloma-to-Carcinoma Sequence*

The occurrence of malignant epidermoid cancers at the base of induced papillomas is, through its implications to the cancer process, the major problem associated with this tumor virus. At first the transformation was thought to be limited to the induced lesions of domestic rabbits, but Syverton (1952) found: (1) it was not uncommon in Western cottontails carrying naturally occurring papillomas; (2) the rate of transformation in these rabbits was similar to the rate displayed by Eastern cottontails bearing induced papillomas; (3) the tendency toward the development of malignancy was most pronounced in domestic rabbits. It will be recalled that in these three groups of rabbits Syverton found a similarity in the persistence of papillomas in Eastern cottontails and domestic rabbits, whereas the papillomas of Western cottontails showed a greater tendency to regress. The fact that the Eastern cottontails resembled domestic rabbits in the persistence of their papillomas, but resembled Western cottontails in the ability of their papillomas to undergo the change to malignancy, suggests the importance of genetic factors in the reactions between host and virus.

Efforts to incriminate the virus in the change to malignancy were difficult for the simple reason that it was not detectable in the cancer. Rous (1936)

suggested that the increased proliferative capacity of the cell in which the virus was enclosed provided an environment conducive to the emergence of a variant virus capable of initiating the neoplastic transformation. This variant was not destroyed by the host's antibodies because of its protected position in the cell, but was limited to the transformed cell and its descendants. As time passed, this concept of a masked virus became of major importance to the tumor virus problem and has been invoked to explain unsuccessful attempts to expose viruses as causative agents of tumors. It has become more popular during recent years, following the discovery that some viruses, after gaining access to cells, enter into a vegetative stage where they are not detectable, but later may reappear in the infective stage, which represents the recognizable and familiar virus particle. Noyes and Mellors (1957) have interpreted their recent findings with the papilloma virus as being in accord with this theory. They applied the techniques of immunology and fluorescence microscopy to ascertain the cellular localization of the papilloma virus in lesions from naturally infected cottontails and from laboratory-infected domestic rabbits. In cottontails, the virus was found only in the nuclei of epidermal cells and in highest concentration within differentiating cells. It was not found in the layer of deep germinal cells, was abundant in the intermediate keratohyaline layer, and present in lesser amounts in the superficial keratinized layer. In lesions of domestic rabbits only minute amounts of virus were discernible. These findings were consistent with the known characteristics of the virus in these hosts. The investigators suggested that their techniques may have revealed only the complete, or infective stage, virus; it was not found in the deeper proliferative cells because, within them, it may have existed in the incomplete, or vegetative stage, during which it was nonantigenic and, therefore, not demonstrable with the technique.

The assumption of a lasting relationship between an inapparent virus and a cancer cell is provocative and should stimulate investigators to explore the mechanisms of this cell-virus relationship. An alternative assumption becomes available for investigation when it is assumed that a virus may be responsible for the change to malignancy but not be essential for the continuous proliferative power of the cell. The papilloma virus, according to this postulate, induces primary lesions in the epidermal cells of both cottontails and domestic rabbits, but it reproduces more in its natural cottontail hosts. The appearance of malignancy is not, therefore, correlated with virus multiplication because in the cottontails more papillomas regress and fewer malignancies arise. The transformation to the neoplastic state could represent a chance distribution of susceptible cells among the basal cells of the epidermis and the enduring qualities of the papillomas in domestic rabbits makes their cells more prone to modification. Briefly, differences between the genetic compositions of the cells of cottontail and domestic rabbits are responsible

for their ultimate reactions to a virus capable of initiating cellular proliferation. The virus plays an important role in the cancer process because it is responsible for the first step in the papilloma-to-carcinoma sequence.

Various chemical carcinogens produce papillomas in the rabbit and in other rodents and, if applications of the carcinogen are continued, some of the papillomas become malignant. This is relevant to the present discussion because the necessity of an enduring relationship between the cell and carcinogen is not essential for the propagation of these cancers in new hosts. The supposition that some tumor viruses, and the papilloma virus could be one, are responsible only for the initiation and not the completion of the cancer process is attractive because it implies that ordinary viruses may be implicated in the origins of tumors. This idea may have led Duran-Reynals (1952, 1957a,b) to investigate the neoplastic properties of the viruses of fowlpox and vaccinia. In the final analysis, a cancer cell is a modified cell and it is becoming increasingly clear that this modification is dependent upon the interplay of various influences. Hence, one of the major problems of the tumor viruses is to ascertain their roles in relation to other known influences which bear upon the cancer process.

One experimental finding in support of the concept that virus must be present to assure continuous propagation of a virus-induced tumor was the demonstration of antibodies in rabbits bearing transplants of the V_2 carcinoma (Kidd, 1942). This tumor was induced in a domestic rabbit with the papilloma virus and throughout a series of transfer generations all efforts to expose the infectious virus were unsuccessful, but antibodies to the virus were detectable in the sera of the hosts. This was interpreted as evidence that the virus was present in the tumor but in a noninfectious, or vegetative, form, until it was found (Rous *et al.*, 1952), during subsequent generations, that antibodies to the virus were no longer present and the tumor remained propagable. Perhaps the simplest explanation for the findings in earlier transfer generations would be that the virus was a "passenger" in the tumor instead of an essential "masked virus."

Beard (1956) has assembled impressive evidence against the theory of the masking phenomenon in virus-induced tumors and used the rabbit papilloma virus as the test virus for his viewpoint. His discussion was based upon the acquisition of highly purified preparations of the virus (Beard *et al.*, 1955) and known host-virus relationships. Use of purified virus in quantitative studies of the host response permitted an estimation of the number of virus particles obtained from papillomas and the number necessary to induce papillomas. The yield of virus from cottontails varied 1000-fold and from some no measurable amounts of virus were recoverable. The maximum computed yield from domestic rabbits was much less; the best from their lesions was only 0.48 % of the best from cottontail papillomas. Calculations

of the amount of virus necessary to provoke lesions in most susceptible rabbits and a comparison of these calculations with the amounts of virus recoverable from domestic rabbits indicated there was an insufficient quantity of virus in papillomas of domestic rabbits to induce growths in new hosts. Thus, the noninfectiousness of material from papillomas of domestic rabbits reflected the small quantity of virus they contained, and was not related to a change in the quality of the virus. He exposed the weakness of serological studies interpreted as bolstering the theory of masked virus, by computing the amount of purified virus necessary for the production of antibodies and showing that, in the domestic rabbit, this quantity was considerably less than the amount required to induce papillomas.

The reader must read Beard's publication to judge for himself the validity of the evidence. Beard may be open to the criticism that his conclusions concerning the infectiousness of the virus include only the infective stage of the virus, but his use of serological studies to refute the theory is challenging. Regardless of the final outcome of the controversy, Beard has established the point that the acquisition of purified virus preparations and the use of such materials in quantitative studies of the host-virus relationships are essential before a qualitative change in the virus is accepted as the causation of unexplained findings. Finally, he focused attention upon the host and stated: "The influence of the genetic status of the host, with respect to the individual, to the strain, and to the species, on tumor virus infection and multiplication can scarcely be overemphasized."

It is safe to assume that further investigations with this virus concerning the "masking" phenomenon will contribute to the knowledge of tumor viruses. Tissue cultures of papilloma and carcinoma cells could be compared for the presence of virus, and the ability of the virus to transform normal or papilloma cells to malignant cells *in vitro* could be explored. Other avenues of approach are open and these will certainly be pursued because of the recent interest in the viral etiology of tumors.

Before closing this discussion, it is deemed necessary to mention two other major contributions to the tumor problem resulting from work with this virus. These have been overlooked because of the position the virus holds in the dispute over the "masking" phenomenon. The papilloma virus was the first tumor virus to be purified. Its presence in large quantities in keratinized papilloma cells of cottontail rabbits simplified the problem of isolation (Beard *et al.*, 1939). Of more interest to the problems of the tumor viruses was the use of the virus for first studies of host-virus relationships in which biomathematical procedures were used (Bryan and Beard, 1939, 1940a,b,c). The application of newer analytical methods to the tumor viruses has done much to extend knowledge of the properties of the viruses and to clarify the problems of host-virus and cell-virus interactions.

D. Fibroma of Rabbits

A fibroma of rabbits, also discovered by Shope (1932a), was shown to have a virus as the causative agent and this virus became an important tumor virus when Shope (1932b) found it was related to the virus of rabbit myxomatosis. The fibroma virus will be discussed briefly because reviews by Smith (1952) and by Oberling and Guérin (1954) contain all essential information, with the exception of recent findings with tissue culture techniques.

The disease is characterized by the occurrence of subcutaneous masses consisting of cells resembling fibroblasts, interspersed with collagenous fibrils and inflammatory cells. Natural transmission through contact is suspected but not proved; Kilham and Dalmat (1955) were able to demonstrate insect transmission of the disease under laboratory conditions.

The virus can be transmitted to domestic rabbits from which, in contrast to the virus of rabbit papillomatosis, it can be recovered from the tumor, blood, spleen, and other organs. The resultant tumor is benign and regresses within 4 to 5 weeks in domestic rabbits, but persists longer in wild hosts. The virus is very labile and distinct strains have been procured during the course of experiments designed to ascertain its properties (Smith, 1952). Local irritants accelerate the occurrence of tumors and, when the virus is injected intravenously, it may induce a generalized fibromatosis. Duran-Reynals (1940b) produced a generalized disease resembling myxomatosis in very young rabbits by the administration of a large amount of virus.

Kilham (1955) discovered a similar disease in squirrels which, when transmitted to suckling squirrels, produced generalized lesions but, when transferred to adult squirrels, produced local temporary lesions. Neutralization tests suggested a relationship between the squirrel and rabbit viruses.

The most interesting property of the virus is its relationship to the virus of rabbit myxomatosis. Animals immune to the fibroma virus are resistant to contact infection with the highly fatal myxoma virus and serological studies with the viruses reveal similar and dissimilar antigens. This suggests that both viruses could have a common ancestor which, it will be recalled, has also been postulated for a variety of chicken tumor viruses. The rabbit and squirrel viruses extend this concept to the inclusion of other species. Evidence at hand, however, permits only the speculation that some tumor viruses are able to produce the natural disease in different species. The occurrence of fibromas in different species and their suggested relationships should encourage further investigation.

An important and stimulating discovery with the fibroma virus was reported by Berry and Dedrick (1936a,b) who found that the inoculation of domestic rabbits with a mixture of fibroma virus and heat-inactivated myxoma virus produced myxomatosis in the test animals. This indicated that the inactivated myxoma virus contained a substance capable of transforming the fibroma

virus from a benign tumor virus to a virus which was highly infectious and produced a fatal disease. The implication of this basic observation is that any virus which, under ordinary conditions, elicits a mild degree of cellular proliferation, may, when exposed to a suitable environment, be transformed to an active tumor virus. Smith (1952) has reviewed the literature stemming from the work of Berry and Dedrick, including her own efforts, and came to the conclusion: "The transformation phenomenon was troublingly irregular." She analyzed the factors involved and, among others, found: "Host factors are at work of which we have as yet no clear understanding." The problems of the fibroma virus, as well as those of the papilloma virus, have been difficult because hosts of known genetic constitutions were not available.

Kilham (1957, 1958) has recently applied tissue culture techniques to a study of the transformation of fibroma into myxoma virus and obtained interesting results. He added live fibroma virus and heat-inactivated myxoma virus to cultures of rabbit testes or kidneys and obtained myxoma virus from the cultures. Later he found that the transformation occurred in most experiments in which cultures of rabbit kidney were used and in 4 of 10 experiments in which cultures of monkey kidney were used. Of interest was the transformation of squirrel fibroma virus to rabbit myxoma virus in tissue cultures.

Kilham's findings are important because they not only indicate a reliable procedure for reproducing the transformation but they also suggest that the application of tissue culture techniques to the problems of the tumor viruses may enable the investigator to avoid the disadvantages encountered in the use of test animals of unknown genetic composition. It is known that cells in tissue cultures thrive in body fluids from heterologous animals and perhaps, in the same environment, cell-virus reactions occur which are prevented *in vivo* by the natural resistance of the host. Recent papers by Andrewes and Chaproniere (1957) and Chaproniere and Andrewes (1957), who used the viruses of rabbit myxoma and fibroma, contain convincing evidence that viruses specific for one species do multiply in tissue cultures of other species.

The major contribution of the rabbit fibroma virus to the tumor viruses has been the important implication that viruses responsible for the production of benign tumors deserve as much consideration as those responsible for malignant growths.

E. Mammary Cancer of Mice

The discovery of a virus as a causative factor in the occurrence of mammary cancer in inbred mice was one of the most fascinating developments in the field of virology. The sequence of events leading to its discovery and subsequent investigations with the virus have produced results of invaluable importance to the tumor viruses, because the relationships between the

virus and its hosts were found to be so different from those of most known tumor viruses that the scope of virus-induced tumors could be enlarged to include almost all tumors.

Cancer of the mammary gland in mice had been studied intensively for many years. It was the tumor of choice because its external location made detection easy and it occurred frequently in a common laboratory animal. Its tendency to grow progressively, to recur after incomplete removal, and to metastasize established it as a malignant tumor (Dunn, 1953). The fact that a mammalian tumor of common occurrence had been characterized as a malignant neoplasm before a virus was implicated in its origin made a profound impression upon cancer workers. This was the first major contribution of the virus to the cancer problem.

Before the discovery of the virus, the occurrence of breast cancer in mice was known to be dependent upon the susceptibility of the animal and the effects of hormonal stimulation. The relative importance of these factors was difficult to ascertain and, to provide suitable test animals, inbred strains of mice were established to fulfill the requirements of controlled genetic investigations. Through the process of selection, strains were procured which showed high or low incidences of breast tumors. When reciprocal breeding between such strains was carried out by the staff of the Roscoe B. Jackson Memorial Laboratory (1933) and by Korteweg (1934), it was found that mice born to high-tumor strain mothers developed mammary cancers while those born to low-tumor strain mothers did not. This showed that the maternal parent was largely responsible for the occurrence of tumors in the offspring; by foster-nursing procedures, Bittner (1936) found that mother's milk contained an agent which was responsible for the production of tumors. For the purpose of this discussion it is essential to note that discovery of this virus followed the development of inbred strains of mice showing extreme variations in their susceptibilities to the development of breast cancer. Briefly, discovery of the virus was a by-product of an effort to ascertain the influence of the host upon the occurrence of this tumor.

The importance of this virus as a member of the tumor viruses has been emphasized repeatedly by every investigator who has reviewed the subject since 1936. The entire problem of mammary cancer in mice was presented in a symposium (1945) by members of the staff of the National Cancer Institute. Other reviews dealing specifically with the problem have been prepared by Bittner (1948, 1955, 1957a), Andervont (1946, 1949a, 1955), and Dmochowski (1953a, 1957). The reader is referred to these earlier summations for details of work with the virus. This discussion will include those facets of the problem which are receiving most attention and, for the most part, only recent publications. Almost all experiments with this tumor involved studies of the host-virus relationships and, although it is impossible to draw clear lines of

distinction between such efforts, this presentation is divided into three parts, namely, host-virus relationships as they concern; (1) the role of the virus in producing tumors; (2) the latency of the virus; (3) the age factor in response to the virus.

1. *The Role of the Virus*

The production of any virus tumor is the result of interactions between the host, the virus, and the environment; breast cancer in mice is no exception. Discovery of the virus added another such influence to those of heredity and hormonal stimulation. Genetic factors control the degree of susceptibility of mammary tissues to the virus as well as the ability of the mouse to propagate the virus. They also control the susceptibility of mammary tissue to hormonal stimulation and may modify the hormonal stimulation of the host. Hormones exert a pronounced influence through their control of the development of mammary glands and thereby provide a substrate for the action of the virus; mammary tumors do not appear in mice in the absence of estrogenic stimulation.

All three factors, genetic, hormonal, and viral, are known to be important and it is the consensus that a tumor can be produced when a deficiency in one factor is compensated by an increase in one or both of the other factors. Other influences, such as diet and overcrowding (Andervont, 1944), are known to affect the occurrence of the tumors. This attitude is a natural outgrowth from previous acceptance of the importance of hereditary and hormonal influences in the appearance of the tumor before the virus was exposed. The important point is that, with this virus-induced tumor, the virus from the first has not been the sole object of attention; instead, it is considered as part of a complex interplay of forces leading to the production of cancer. And this is important because, with the acceptance of the virus as but one factor in the cancer process, the virologist enlarges his viewpoint of the viral etiology of cancer. The virus of mammary cancer in mice was not discovered by virologists, but by geneticists who were interested primarily in an evaluation of the genetic factors concerned with the occurrence of the tumor.

In recent years, even the necessity of the virus has been seriously questioned, and, to date, most evidence supports the idea that mammary tumors can arise in mice in its absence. In an early review, it was pointed out (Andervont, 1945a) that mammary tumors occurred in a few hybrids derived by mating low-tumor strain females to high-tumor strain males, but the incidence of such tumors was very low. An opportunity to investigate the problem presented itself when it was found that first generation hybrids born to low-tumor strain BALB/c females and high-tumor strain C3H males showed a high incidence of mammary tumors (Andervont, 1945b). These tumors were

histologically similar to those procured with the virus but arose in mice at a later average age than did the virus-induced tumors. Hybrids were obtained from mice of various inbred strains which were presumably free of the virus, those derived from strains susceptible to the virus showed the highest incidences of tumors (Andervont and Dunn, 1948a). In the discussion of these findings it was suggested that the virus could act as an accelerator in the production of tumors because, in the agent-free hybrids, tumors arose in mice of certain genetic derivations and only after they had experienced considerable hormonal stimulation.

Heston *et al.* (1950) reported a relatively high incidence of breast tumors in members of a substrain of strain C3H mice which were free of the agent. This substrain was procured by removing a litter by cesarean section from a high-tumor strain C3H female and foster-nursing them upon a low-tumor strain C57BL female. The first five generations of the substrain were bred intensively and showed a mammary tumor incidence of 38 % at a mean age of 20 months. Further, mice with tumors were not members of certain families which would be expected if the virus had gained access to a few females. It was concluded that the tumors arose in agent-free mice because of their genetic composition and intensive breeding.

Additional evidence of breast tumor production in virus-free mice was disclosed when they were used as experimental animals in investigations involving the occurrence of breast tumors in mice subjected to skin painting with the carcinogenic hydrocarbon methylcholanthrene. Mider and Morton (1939) applied the carcinogen to the skin of virus-carrying strain DBA mice and found that the painted mice developed mammary tumors much earlier than did control animals. Later, Andervont and Dunn (1950) and Bittner and Kirschbaum (1950) used the same technique for producing breast tumors in virus-free DBA mice and did not find the virus in the induced tumors. Dmochowski and Orr (1949) obtained similar results, but with mice of strains IF and C57BL, which were relatively resistant to the virus.

Despite evidence that the virus was not involved in the occurrence of these mammary tumors, Dmochowski (1953b) questioned this interpretation after an extensive investigation in which hybrids derived from virus-free C57BL females and virus-containing RIII males were used. The progeny of some of the female hybrids developed mammary tumors and, of 23 such tumors assayed for the virus, 17 were found to contain it. Dmochowski discussed his findings in relation to those of others and suggested: "The conclusion that tumors in old hybrids do not harbour the agent and are the result of a combined action of hormonal and genetic factors only should at least for the time being be suspended, in view of the discovery of the agent in some mammary tumors appearing at an older age than 15 months . . ."

Heston (1958) and his colleagues (1956; Heston and Deringer, 1952) have tried repeatedly to expose the virus in their virus-free substrain of C3H mice but with negative results. Hybrids procured by reciprocal mating between their C3H substrain and strain C57BL showed similar incidences of tumors; if the C3H animals carried the virus, then their offspring should develop more tumors than those derived from the agent-free C57BL mothers. One exhaustive experiment consisted of outcrossing virus-carrying strain C3H females to virus-free C57BL males and then backcrossing the female offspring to C57BL males for seven generations. The virus was eliminated by the third backcross generation. When the seventh backcross generation females were bred to virus-free substrain C3H males and their female progeny backcrossed to similar males for four generations, the concentration of strain C3H chromatin did not bring about the reappearance of the virus. This showed that the virus was not present in the last generation of backcross mice born to C57BL fathers for, had it been present, the concentration of chromatin from highly susceptible C3H males would have permitted it to increase in amounts sufficient to produce tumors. Finally, a thorough analysis of the tumors occurring in the virus-free substrain gave no indication of the presence of the virus. For example, females with three successive generations of tumors in their immediate maternal ancestry did not develop more tumors than those with tumor-free ancestors. Instead, analysis revealed a correlation between the numbers of litters the females bore and their incidences of tumors.

Andervont and Dunn (1958) have continued their studies with hybrids by the administration of estrogenic stimulation to castrated males derived from virus-free parents. Some groups of these male hybrids showed tumor incidences ranging from 59 to 84 % at average ages of 12 to 14 months and, as in the earlier work, hybrids most susceptible to the estrogen-induced tumors were derived from inbred strains which were most susceptible to the virus; hybrids with a strain C3H parent were among the most susceptible.

Boot and Mühlbock (1956) have reviewed the literature dealing with the production of mammary tumors in virus-free inbred mice and have presented their findings with virus-free strain C3H animals. They arrived at the conclusion that the occurrence of mammary tumors in these mice resulted from a combination of hormonal stimulation and a high susceptibility to mammary cancer and, "the only logical explanation of this susceptibility seems to be to consider it as an expression of the genetic constitution of the agent-free C3H substrains." Mühlbock (1956), in a recent review of the hormonal genesis of mammary cancer, records the production of mammary tumors in virus-free mice of strains C3H, DBA, O_{20}, and C57BL by subcutaneous transplantation of hypophyses. The successful induction of mammary tumors in virus-resistant C57BL animals is conclusive evidence that these tumors do arise in the absence of the virus.

It is essential to mention that the great majority of experimental results mentioned above were carried out with inbred mice which were highly susceptible to the virus and in which the virus is transmitted from generation to generation. These investigations were not performed with animals of unknown genetic composition and there is every reason to assume that a small amount of virus would produce tumors in them and, furthermore, that in successive generations the virus would increase sufficiently to produce tumors. The only conclusion permissible at present is that the weight of evidence favours the occurrence of mammary tumors in virus-free mice.

If the virus is not essential for the production of all breast cancers in mice, then studies with this virus have made another significant contribution to the problems of the tumor viruses, for it becomes clear that the discovery of a viral etiology for a tumor does not prove the virus is responsible for all other tumors of the same type. Other viruses or no virus may be involved in these latter tumors. This makes the search for tumor viruses more difficult because they may play a minor part in the complicated interactions of various influences resulting in malignancy.

2. *The Latency of the Virus*

If the term latent implies the presence of a virus in the host without the production of the disease, then the mammary tumor virus of mice certainly has a long latent period. A newborn mouse need remain with its mother for only a few hours after birth to acquire sufficient virus for the occurrence of a tumor in middle or late life. Further, during this time, while the mice are without tumors, the virus is detectable in tissues of infected animals and is always present in the milk. This unique property of the virus made possible its discovery, for geneticists had developed inbred strains of mice with varying degrees of susceptibility to the development of mammary cancer before the virus was known. After the virus was found, it was learned that all high-mammary cancer strains carried it whereas low-mammary cancer strains did not. Selection toward susceptibility to the tumor was also selection toward susceptibility to the virus; this implies that the genetic make-up of the host may be of much importance in the activation of a latent virus.

At least one inbred strain was established which did not develop tumors but was susceptible to the virus; when mice of strain BALB/c were suckled by foster mothers from strain C3H that carried the virus, the BALB/c strain was changed to a high-tumor strain (Andervout, 1945c, 1949b). The ability of strain BALB/c to propagate the virus became of more interest when it was discovered, as stated previously, that hybrids derived from presumably virus-free BALB/c females and strain C3H animals showed a high incidence of mammary tumors (Andervont, 1945b). It was concluded that the tendency

to develop tumors was either a unique characteristic of these hybrids or the strain BALB/c females carried a latent or masked virus; the latter possibility appeared likely in view of the susceptibility of these mice to the strain C3H virus. A series of investigations was performed to reveal the presence of a mammary tumor virus, or a variant therefore, in strain BALB/c mice. These studies are not reviewed because a publication by Andervont and Dunn (1953) contains all relevant references. It was concluded that the virus was not responsible for the high incidence of tumors in old hybrids obtained from BALB/c females and that these tumors resulted from the interplay of hormonal stimulation and hereditary factors in the absence of the virus.

A deliberate attempt to mask and then unmask the virus has been made with the virus-resistant C57BL strain. These mice were suckled by strain C3H females and by suitable procedures it was found that the virus had disappeared from them within a few generations (Andervont, 1945c). Descendants of these mice have been maintained for more than twenty generations of inbreeding and repeated efforts to expose the virus in them have been unsuccessful.

The virus appears to be very stable once it is established within an inbred strain, but the literature does contain a few references to its sudden appearance or disappearance. Bittner (1941) described the occurrence of a mammary tumor in a strain A mouse whose ancestry for seven generations did not develop this type of tumor but whose descendants for four generations showed a high incidence of tumors. Bittner suggested that the milk of mice may contain an inactive virus which may change to an infectious virus or the infectious virus may arise *de novo* within a mouse. Murray and Warner (1947) watched for and observed the disappearance of the virus from a mouse of the Marsh strain. They had observed previously a progressive decrease in the tumor incidence of the strain and had attributed it to the presence within the strain of a family which did not develop tumors. Starting with a pair of mice whose ancestry showed a high incidence of tumors, they observed that neither one of the daughters nor any of her 351 female descendants developed a breast tumor. After analyzing all the available data, the conclusion was reached that the Marsh strain females transmitted variable amounts of virus, or an altered virus, and the variation in the quantity or quality of virus remained consistent through later successive generations. This interesting paper has been overlooked by most reviewers of the mammary cancer virus.

For many years the writer has maintained inbred strains of mice and has watched carefully for any evidence of contagion and for the sudden appearance of the virus. Strains free of the virus but highly susceptible to it have been housed close to strains which carried it and the former were observed constantly for any sudden acquisition of the virus. This has not occurred but,

within the past few years, the virus suddenly disappeared from two members of the RIII high-cancer strain (Andervont, 1958). The disappearance was observed within two generations of its occurrence because of the high incidence of tumors in the strain, and close descendants of the mice from which the virus disappeared were available for study. This finding has confirmed the observation of Murray and Warner.

The chief reason for mentioning these deviations in the activity of the virus is to draw attention to the fact that they occur rarely in inbred mice. This could almost be expected because, similar to the Rous virus, the virus of mouse mammary cancer has not only been maintained in the laboratory for many years, but its hosts have been inbred intensively for susceptibility to its activity. Perhaps the establishment of inbred strains by selection toward susceptibility to the virus may have resulted in the unique host-virus relationship which permits detection of the virus long before the host has cancer.

In order to ascertain whether this relationship was restricted to inbred hosts and, also, whether the virus was involved in the natural disease, it was thought advisable to search for the virus in wild house mice. The necessity for the latter study stemmed from the knowledge that tumors arose in inbred mice and in their hybrids in the absence of the virus. Two reports (Andervont, 1952; Andervont and Dunn, 1956) contain details of the methods used and the results obtained in this investigation. The virus was exposed in wild house mice by having them suckle virus-free inbred mice which were very susceptible to the virus. The occurrence of tumors in the inbred mice indicated that the wild mouse virus was low in activity or in amount because the fostered animals developed few tumors. Wild mice suckled by inbred mice carrying a potent virus did develop tumors but showed a lower incidence than inbred mice exposed to the same virus. These findings permitted the conclusion that the wild house mice carried a virus of low activity which was transmitted through successive generations of relatively resistant wild mice. Recent studies (Andervont, 1958) have shown that the wild mouse virus increased in activity after passage through twenty generations of inbred mice. Thus, three strains of the virus are available: one, low in activity or amount and carried by wild mice; another, of medium activity after twenty generations of passage in inbred mice; another, the more familiar virus, of high activity after many years of passage through inbred animals. It would appear that the strain most suitable for exploring the problems of latency and masking is the one carried by wild mice. Present knowledge of the strains indicates that the ability of resistant wild mice to maintain the virus may be attributed to their mixed genetic constitutions, whereas, with the inbred strains, the virus has attained such a high degree of activity that it is easy to detect, and masking, if it ever occurs, is an exceedingly rare event.

These virus strains, especially the strain harbored by wild mice, may prove useful in an immunogenetic approach to the problems of host-virus interactions. Antibodies to the virus have not been found in mice of susceptible inbred strains. It is conceivable that years of intensive and selective inbreeding, concurrent with the acquisition of a highly active virus, could account for the inability of inbred hosts to produce antibodies against the virus. In the wild mice of mixed genetic composition the virus may elicit antibodies in some individuals but not in others; this could explain why, in general, these mice are relatively resistant to the virus but are incapable of destroying it. An explanation of the reason for the persistence of the virus in the resistant house mouse should be of interest to the tumor virus problem.

3. *The Age Factor in Response to the Virus*

The age of the mouse is an important factor in determining its susceptibility to the virus because very young animals are more susceptible than adults (Andervont, 1941, 1945d). It has even been suggested that the virus cannot produce tumors unless it infects mammary glands early in their development. The necessity of using very young test animals, and the long period of time between infection and the appearance of a tumor, together with the occurrence of tumors at sites remote from the site of virus entry, are undoubtedly the chief reasons for the delayed discovery of the virus. These factors were far removed from any previous experience which revealed a virus as the causative agent of a tumor.

Hybridization between inbred strains led to the discovery of the mammary tumor virus and the same procedure produced the first evidence that adult mice were susceptible to infection. Hybrids were derived by mating virus-free females to virus-bearing males and some of the offspring developed tumors at an early age and carried the virus. It was suggested (Andervont and Dunn, 1948b) that the hybrids had acquired their father's virus because virus was found in the seminal vesicles of high-mammary tumor strain males (Andervont and Dunn, 1948a). Subsequent work in various laboratories established transmission of virus to the female during copulation. This interesting problem has received attention from Bittner, who has summarized most of his findings in a recent publication (Bittner, 1957b). Dmochowski (1953a) has reviewed the entire problem.

It is not clear how the father's virus gains access to the hybrid offspring. In most instances the mother is infected first and then transmits the virus to the young mice in her milk, but in a few litters some hybrids developed tumors at an early age and these tumors contained the virus, whereas other litter mates live to an advanced age and either die free of tumors or develop tumors in which the virus is not detectable (Andervont, 1949a).

Such finding suggest that a few hybrids may have acquired their father's agent while *in utero* and this reminds the virologist of the "scrapie" virus of sheep which can be transmitted by the sperm to the offspring without infecting the mother (Greig, 1940).

The routes of transmission whereby the progeny get their father's virus is not as pertinent here as is the fact that the mothers are infected. These females may or may not develop tumors but in either event they do transmit the virus to their offspring and this revealed an unexpected path for virus transmission. Persistent investigations are essential before the epidemiology of the tumor viruses can be learned.

The problem of how the virus infects adult females is unsolved. The placenta is an effective barrier against transmission of the virus from an infected mother to her unborn offspring. Perhaps the genital tract is receptive during a stage in the estrous cycle. If the mother is infected during copulation the virus must increase rapidly to infect her milk because the occurrence of tumors in a single litter is used to ascertain when the mother acquired the virus (Bittner, 1957b). The important thing is that the virus not only infects adult females, but apparently propagates rapidly in them. The mechanisms responsible for this interaction deserve consideration.

Infection of adults suggests further investigations of a problem which is of interest to virologists. The mammary tumor virus, in contrast to other tumor viruses, appears to be incapable of eliciting antibodies in its natural hosts but does so in heterologous hosts. The absence of demonstrable antibodies in mice carrying the virus may be the result of exposure to the virus shortly after birth, when they are known to be in a state of immunologic nonreactivity. Adults may be reactive and respond to the virus by the production of antibodies. Disappearance of the virus from mice of the Marsh and RIII strains suggests that the genetic constitution of the test animal may play an important part in immunological responses to the virus. The inability of the virus to propagate in all strains of mice suggests the presence of a resistance factor; the elucidation of the nature of this factor presents another interesting problem.

Anyone familiar with this tumor virus will realize that this discussion includes only a few of the problems now receiving consideration. From the first, one of the most important problems has been the development of a technique for detection of the virus which will dispense with the months of waiting for the appearance of tumors in test animals. A method for rapid quantitative assay of the virus is also desirable because the known properties of the virus make it almost ideal for quantitative studies of the host-virus relationship.

A satisfactory assay technique could also assist investigations of the cell-virus relationships. The virus appears to be very specific for mammary gland

cells and the only known lesion indicative of its presence prior to the appearance of neoplasia is the early occurrence of hyperplastic nodules in the glands. The observations by Jones (1956) that hyperplastic nodules are also numerous in the glands of old mice free of the virus calls for further exploration of the possibility that the virus acts as an accelerator in the production of tumors. If it can be established that the role of the virus in the production of the tumor is to provide these nodules in which the tumor develops, then the virus has done much to further knowledge of the tumor viruses, for this would imply that any virus capable of producing benign hyperplasia could be implicated in the origin of cancer.

A brief survey of the literature produced only one reference to the application of tissue culture techniques with the virus. Pikovski (1953) reported the survival of the virus for 5 months in tissue cultures of chicken fibroblasts. Tissue cultures of mouse embryos may provide an excellent substrate for cultures of the mammary tumor virus.

The major contribution of this virus to the tumor viruses has been the establishment of the importance of the host in any host-virus relationship. Regardless of whether the virus is found to be the direct cause of the tumor or whether it acts indirectly by producing a benign lesion which, under suitable environmental conditions, proceeds to malignancy, the genetic constitution of the host is of much importance in the sequence of events which terminates in cancer.

F. Leukemia of Mice

Since 1951, an effort to establish a causal relationship between a virus and leukemia of mice has been the most interesting, exciting, and confusing feature of the tumor viruses. It has been the most interesting and exciting because oncologists familiar with the characteristics of mouse leukemia and its widespread occurrence in all inbred strains have seriously considered the possibility that a virus could be involved. The establishment of strains showing high or low incidences of spontaneous leukemia, together with experience gained from studies of mouse mammary cancer, made possible a rational approach to the problem.

As the work progressed it became clear that the task was not easy. It became very complex when the test animals developed a variety of tumors which were not characteristic of leukemia. This difficulty had not been encountered with other virus-induced tumors which were reproducible by the administration of filtrates to susceptible tissues of suitable hosts. Furthermore, those who entered the field published contradictory and, at times, confusing results. A review of the literature gives the impression that the co-operation and free exchange of information maintained by early investigators who worked with the mammary tumor virus has, thus far, been lacking in studies of leukemia.

For the object of this discussion, these factors make it difficult to present a review which most readers can easily understand. Perhaps the best approach is to follow the experiments reported by Gross, who supplied recent evidence for the viral etiology of mouse leukemia, and to refer to others when their results are applicable to his findings. It is hoped that this procedure will assist the reader in focusing attention upon separate facets of the problem at different times and avoid the confusion which usually prevails when the work is presented chronologically.

1. *Leukemia*

The first publication by Gross (1950) described experiments in which mice of the inbred strains C3H and C57BL were inoculated subcutaneously with cell suspensions of leukemic tissues from strain AK/n mice[1] bearing a transplantable leukemia. The tumor grew in some recipients of both strains, but 1-to 7-day-old C3H and 1-day old C57BL mice were far more susceptible than older mice of the same strains and these latter animals displayed an increasing resistance with increasing age. Gross concluded with the statement: "It remains to be seen whether all these mice . . . that did not apparently react to the inoculation of the leukemic-cell suspensions will remain free from leukemia for the balance of their lives." Gross (1952a) supplied the final information concerning strain C3H only. Of 62 mice that had received cell suspensions when 8 to 60 days old, 25, or 40 %, developed leukemia between the ages of 13 and 24 months without any evidence of tumors at the site of the initial inoculation.

The occurrence of leukemia in these animals is of considerable interest because in most of his later papers Gross emphasized the importance of using newborn mice (less than 16 hours old) for the detection of the virus, although in his earlier work (Gross, 1951b, 1952b) mice up to 8 days of age were susceptible. He used 43, 22, and 7 mice when they were 2–7, 2–6, and 8 days old, and 11, 12, and 2, developed leukemia at average ages of 17.6, 17.8, and 25 months, respectively. It is difficult to correlate these findings with his statement a few years later (Gross, 1954a) that: "Newborn mice less than 16, or better, less than 12 hours old had to be injected in order to successfully transmit the leukemic agent and cause the development of leukemia in the inoculated animals. When older mice were inoculated, the results were either negative, or considerably delayed and erratic." It is true the results were

[1] The standardized nomenclature for inbred strains of mice and their hybrids, prepared by a committee (1952) appointed for this purpose, is used throughout this chapter. The designation AK/n refers to a line, maintained by Gross, of the high-leukemia AKR strain. When authors refer to their mice as the AK strain, the writer used his best judgment in the use of the AKR and AK/n designations.

First generation hybrids derived from two inbred strains, such as strain C3H and strain AKR, are designated as (C3H × AKR)F_1 hybrids.

delayed but, according to the published data, they were neither negative nor erratic.

Throughout the remainder of this discussion, unless stated otherwise, all mice were less than 24 hours old when used as test animals for the activity of tumor or normal tissue extracts.

Gross' (1951a) first evidence of a leukemia-inducing agent was obtained when strain C3H mice received supernatant fluids from centrifuged extracts of leukemic tissues from high-leukemia strain AK/n mice. Fourteen mice were inoculated and 7 developed leukemia 8 to 11 months later. Likewise, the administration of cell suspensions of AK/n embryos to 6 strain C3H animals produced leukemia in 4. With this as a start, leukemic tissues from strain AK/n as a source of virus and strain C3H as test mice were used to show: (1) filtrates contained the virus (Gross, 1951b, 1952b, 1953c,d); (2) heated extracts (Gross, 1953a) or filtrates (Gross, 1953c,d) did not elicit leukemia; (3) centrifugation at high speeds (144,000 *g*) sedimented most of the activity in cell-free extracts (Gross, 1953a,d). Additional experiments confirmed the presence of the virus in AK/n embryos (Gross, 1952b). Gross (1953b) used strain C3H as test animals to find the virus in cell suspensions of testes and ovaries from AK/n mice; of 12 inoculated with testicular extracts, 8 developed leukemia, and of 14 inoculated with ovarian extracts, 6 did so.

In a subsequent paper Gross (1954a) summarized his results and compared them with those of others who had studied the virus of visceral lymphomatosis of chickens and, on the basis of his detection of the mouse virus in embryos, testes, and ovaries, discussed the egg-borne transmission of both viruses. He wrote: "It can only be stated that leukemia in chickens or mice, is not transmitted from one host to another in the conventional manner hitherto observed in the common communicable diseases." This statement is quoted because it is relevant to the previous discussion of chicken leukemia in this chapter, and because it is surprising that it was written by an investigator interested in tumor viruses, especially by one (Gross, 1952b) who had recently participated in a conference during which Burmester (1952), in his discussion of the chicken virus, had stated: "This agent is spread and produces the disease by direct contact and probably by the aerogenous route."

Gross (1954b, 1955b, 1956) advanced the problem when he found that the strain AK/n virus evoked leukemia in strain C57BR/cd mice and that centrifuged cell suspensions of leukemic tissues from high-leukemia strain C58 mice were also capable of eliciting leukemia in strain C3H mice. Filtrates of leukemic extracts from C58 mice produced leukemia in strains C3H and C57BR/cd. He also found (Gross, 1956) that cell suspensions of strain C58 embryos produced leukemia in strains C3H and C57BR/cd. These results widened the scope of the studies by showing that his earlier findings could be duplicated with tissues from another high-leukemia inbred strain.

Another advance was made when a strain C58 leukemia was, by means of filtrates, carried through at least 6 serial passages in strain C3H hosts (Gross, 1956). This observation was extended (Gross, 1957a) when a strain AK/n leukemia virus was transmitted through 8 serial passages in C3H mice. The passage virus was procured by using, at each transfer generation, leukemic tissues from the mouse developing the disease earliest. This achievement was important because the virus is now available to other investigators. Gross (1957b) has published a very useful summation of his findings.

The efforts of other workers to confirm the findings of Gross have met with both failure and success. Stewart (1955a,b) injected extracts and filtrates of AKR leukemias into strain C3H mice, but only 3 of 95 animals developed leukemia. Law *et al.* (1955) used the techniques described by Gross and, in 283 strain C3H mice inoculated with extracts of filtrates from leukemic tissues, observed no increase in the incidence of leukemia beyond that of untreated controls.

After Gross had become aware of the results of Law and his colleagues he (Gross, 1955a) re-examined the protocols of his experiments and found a striking difference between the incidences of induced leukemia in substrains of C3H mice he had procured from the National Cancer Institute (NCI substrain) and from the Bittner substrain; 320 of the Bittner substrain showed an incidence of 28 %, and 162 of the NCI substrain showed an incidence of only 4 %. This explanation was accepted as the reason for the different findings reported by Stewart and Law, who used NCI mice and those of Gross. Supporting evidence became available when Woolley and Small (1956, 1957) confirmed substrain differences in susceptibility to the virus and also confirmed Gross by using the Gross subline of Bittner mice. Hays *et al.* (1957) have confirmed the results of Gross by using the Gross subline of strain C3H and also strain C57BR/cd mice. Law (1957) investigated the origins of different C3H substrains and found that the NCI strain has shown a low incidence of leukemia since its separation from the parent strain in 1930; the highest reported incidence was 10 % (Dunn, 1954). The Bittner substrain originated in 1931 and Gross procured his subline from Bittner in 1944. Differences between the various substrains of strain C3H have been recognized and ascribed to genetic differences (Law, 1957).

Law (1957) procured C3H mice from Bittner and observed the occurrence of spontaneous leukemia in them. Gross has repeatedly stated that his subline of Bittner's mice rarely develop leukemia and that the incidence is less than 0.5 %. Woolley and Small (1956) found 2 mice with leukemia in 144 untreated C3H mice of the Gross subline and Dulaney *et al.* (1957) found 8 cases in 83 untreated C3H mice which were descendants of litters procured from Gross. In this report Dulaney also included results attending the inoculation of AKR and AK/n leukemic extracts into C3H mice of the NCI and

Bittner substrains; 17.8 % of the NCI mice and 8.8 % of the Bittner mice developed leukemia. The authors stated that the same extracts were not injected into both substrains and therefore the results should not be compared, but a leukemia incidence of 17.8 % in treated NCI mice was above the incidence of 4 % reported by Gross (1955a).

Further studies with the Bittner substrain mice are necessary to establish the incidence of spontaneous leukemia in them. Leukemia is a common disease in mice and occurs in almost all inbred strains, but to a far greater extent in certain strains, such as AKR and C58. Gross, however, has not limited his use of experimental animals to strain C3H, for, as stated previously, he has established strain C57BR/cd as suitable for confirmation of his findings.

a. Transmission of the Leukemia Virus to Progeny. In one of his early papers, Gross (1951b) reported transmission of the virus from infected parents to their immediate offspring. This aroused considerable interest among oncologists because transfer of a tumor-inducing agent from generation to generation was accepted as good evidence for the presence of a tumor virus. Gross recorded the appearance of leukemia in 9 of 18 offspring born to C3H parents infected with AK/n leukemic cell suspensions and in 4 of 9 offspring born to C3H parents infected with AK/n embryo cell suspensions. While discussing these first findings he stated: "There is no reason to believe that the leukemic agent would stop at this point its vertical trend of transmission." In a later paper, Gross (1952b) reported the occurrence of leukemia in 17 of 46 first generation offspring of infected strain C3H parents. During a brief discussion of the parent-to-offspring transmission he modified his earlier view of continuous transmission by stating: "The possibility, however, must be considered that the leukemic agent may become adapted to the new strain of mice to such an extent that it may, after having passed through one or two generations, fail to become activated even though it may be transmitted through the embryos from one generation to another, and even though it may be carried by the new hosts." This was an unusual interpretation by an oncologist interested in a tumor virus because it placed him in the position of either establishing the absence of an "inactive virus" in his test animals or admitting that they carry an "inactive virus." The widespread occurrence of leukemia in many inbred strains of mice could imply, according to this hypothesis, that all strains carry the virus.

Gross' (1954b) next reference to transmission of the virus from generation to generation consisted of a brief reference to young born to C57BR/cd parents that had been infected with AK/n leukemic extracts; 22 parents produced 147 untreated offspring, of which 10 developed leukemia. The last publication in which Gross (1955b) discussed natural transmission of the virus referred only to C3H mice infected with the virus from AK/n mice.

He recorded a leukemia incidence of almost 50 % in first generation progeny of infected C3H parents but a "rather low" incidence in two subsequent generations. He concluded that his attempt to obtain a high-leukemia line of strain C3H was unsuccessful and, of more interest, modified his original theory of an ever present virus in descendants of infected mice to include the possibility that the virus "may either completely disappear, or it may become so submerged and masked, as to lose, with only occasional exceptions, its ability to become activated spontaneously." It is strange that others who have followed Gross' procedures have not pursued the important implication in his first reports of generation-to-generation transfer of the virus. Dulaney *et al.* (1957), without including data, stated they had not confirmed Gross' observation. Perhaps, as with the virus of mouse mammary cancer, only certain inbred strains will accept and transmit the leukemia virus; it is essential to ascertain whether such strains are available. The transformation of a low-leukemia strain to a high-leukemia strain would help establish the viral etiology of the disease because virus-free mice of the same inbred strain would be available for comparison. If the disease is "egg-borne," as claimed by Gross, then it may be impossible to free infected strains of the virus, and this should encourage the search for other strains capable of transmitting the virus through successive generations. It is difficult to understand why studies with this tumor virus have been limited to only a few inbred strains as test animals when so many are available, and why the techniques for transplantation of ovaries and fertilized ova have not been used more extensively (Fekete and Otis, 1954) to explore its natural routes of transmission.

b. Hybrids as Test Animals. First generation hybrids procured from inbred strains may be useful in studies with any virus. Stewart (1955b) obtained hybrids by mating low-leukemia strain C3H females to high-leukemia strain AKR males and inoculated them with filtrates of AKR leukemic tissues. When the hybrids were 9 months old, 22 of 45 inoculated mice had developed leukemia, in contrast to only 2 of 44 untreated controls. Law *et al.* (1955) inoculated similar hybrids with extracts, centrifugates, or filtrates of leukemic tissues from various sources. They observed, similar to the findings of Stewart, a 4.5 % incidence of leukemia in the inoculated mice before the disease occurred in any of the controls but, at 18 months of age, the incidence in inoculated mice was 18.6 % and in the untreated controls 54 %. Hays *et al.* (1957) have reported interesting preliminary results with (C3H × AKR)F_1 hybrids. They prepared nucleic acid preparations of normal and leukemic lymph glands and spleens from AKR mice and injected them into mice of strains C3H, C57BR/cd, and (C3H × AKR)F_1 hybrids. Preparations from leukemic tissues did not produce leukemia in C3H or C57BR/cd mice, but those from both nonleukemic and leukemic tissues accelerated the occurrence of leukemia in the hybrids.

The use of such hybrids as experimental animals has two serious disadvantages. First, they already carry the virus, if Gross is correct in his opinion that embryos derived from high-leukemia strains are infected. Second, hormones, ionizing radiations, chemical carcinogens, and diet may hasten the time of appearance and increase the incidence of a tumor in experimental animals possessing the predisposition to its spontaneous development. Hence, an accelerated occurrence of the tumor in them is inconclusive evidence of the activity of an extrinsic virus. Suitable hybrids for detection of the leukemia virus should be procured from strains showing low incidences of the spontaneous disease.

In this brief review of efforts to reveal a virus as the causative agent of mouse leukemia no attempt was made to include all the recent literature. It is hoped the reader is aware that time and more work are needed before the viral etiology of the disease is clarified. Gross has shown that a transmissible agent, presumably a virus, is important, but the attempts of others to confirm his results have been conflicting and, to those not directly involved, confusing. The reason for the present state of affairs could be that the investigators, including Gross, have given a large part of their attention to the study of other tumors which arose unexpectedly in the experimental animals. Some of these will now be discussed.

2. *Parotid Gland Tumors*

This tumor was the leading culprit in the diversion of interest, and for good reasons. It arose in mice after they had received extracts of leukemic tissues and was a new type of tumor or, at least, one not recognized previously in the mouse. It has been described adequately by Dunn (Law *et al.*, 1955) and by other pathologists whose descriptions were included in the first few following references to Gross. Considerable space could be given to this tumor but, for the purpose of this chapter, a brief review will suffice.

Gross (1953c,d) recorded the occurrence of parotid tumors in strain C3H mice after they had received centrifuged filtrates of AK/n leukemic tissues or centrifuged extracts from AK/n embryos; C3H mice inoculated with C3H embryo extracts "remained in good health." When AK/n leukemic extracts were centrifuged at 144,000 *g* from 1 to 2 hours, the supernatants and resuspended sediments contained the parotid tumor-inducing agent. Gross (1954b) found that filtered AK/n leukemic extracts did not produce parotid tumors in C57BR/cd mice.

In a subsequent paper, Gross (1955b) gave more information concerning the parotid tumor response of C3H mice exposed to filtrates of AK/n leukemic tissues and extracts of AK/n embryos. He emphasized the fact that parotid tumors never arose spontaneously in his subline of C3H mice. A later publication (Gross, 1955c) included considerable data on the occurrence of

parotid tumors in strain C3H. These mice developed parotid tumors after receiving the following materials: centrifuged or filtered extracts of induced leukemias in strain C3H mice; centrifuged or filtered extracts of induced or transplanted parotid tumors in C3H mice; centrifuged extracts from normal embryos of strain C3H and C57BR/cd; centrifuged extracts of normal lactating mammary glands from strain C3H; centrifuged extracts from pooled normal organs of C3H mice used when fresh, lyophilized, and kept at — 4°C. for ½ to 26 months, or preserved in 50 % glycerine for 2 weeks to 2 years. Gross discussed the possible relationship between the leukemia and parotid tumor agents and reported that only in 3 mice "among several hundred" inoculated did he find both tumors and, in contrast to strain C3H, strain C57BR/cd animals were resistant to the parotid tumor virus. Concerning the development of parotid tumors in C3H mice after they had received materials from other C3H mice he concluded: "This agent would therefore exist in C3H mice in a masked form, usually not pathogenic for its natural carrier." At this point, the writer cannot refrain from suggesting that the same words could be used to explain also the appearance of leukemia in Gross' strain C3H mice until either a high-leukemia strain can be freed of the virus or a low-leukemia strain can be changed to a high-leukemia strain through acquisition of the virus. If the use of the newborn animal is essential for the production of tumors, then the role of the host must be explored before an intrinsic host factor can be excluded as the most important influence in tumor development.

Gross (1956) reported the production of parotid tumors in mice following administration of strain C58 leukemic extracts to different hosts. The results may be briefly stated as follows: Filtrates induced leukemias but no parotid tumors in C57BR/cd mice, but induced both leukemia and parotid tumors in C3H hosts; centrifuged extracts of filtrates of C57BR/cd leukemias induced with C58 leukemias produced leukemia and parotid tumors in C3H mice; similar materials from induced tumors in C3H mice evoked leukemia and parotid tumors in C3H mice; centrifuged extracts from C3H parotid tumors induced with C58 leukemias produced leukemia only in C3H mice, but Gross referred to previous experiments in which C3H materials had induced parotid tumors in C3H mice and considered the small number of animals used responsible for the negative result. In other experiments, centrifuged extracts of strain C58 embryos were administered to strains C3H and C57BR/cd mice; the C3H mice developed parotid tumors as did one of the C57BR/cd mice. This latter tumor represented the only parotid tumor produced in a C57BR/cd mouse in Gross' experience. The injection of centrifuged extracts from normal organs of guinea pigs into 235 C3H mice produced parotid tumors in 4 animals.

As stated previously, Gross (1957b) prepared a summation of his studies in which he reported that filtrates from AK/n spontaneous leukemias produced

parotid tumors in 11 % of C3H mice and cell-free extracts of these induced parotid tumors induced similar tumors in 7 % of other C3H animals.

Other investigators observed the appearance of parotid tumors in their animals while trying to confirm Gross' observations on the detection of the leukemia virus. Stewart (1955a) found them, independently, in strain C3H mice which had received filtrates or extracts of spontaneous or transplanted AKR or AK/n leukemias. It is of interest that Stewart prepared extracts from organs of normal C3H mice and that these extracts failed to produce parotid tumors when injected into other C3H mice. Stewart (1955b) also observed parotid tumors in (C3H × AKR)F_1 hybrids that had received filtrates of tissues from a transplanted AK/n leukemia or from leukemias arising in other (C3H × AKR)F_1 hybrids.

Stewart *et al.* (1957a) exposed tissue cultures of monkey kidney to extracts of parotid tumors and, after a few passages, injected the tissue culture supernatant fluids into (C3H × AKR)F_1 hybrids; of 66 treated hybrids, 34 developed parotid tumors. Cell-free extracts from a transplanted AK/n leukemia and a paraganglioma also induced parotid tumor in similar hybrids. They also administered cell-free extracts from various sources to strain C3H mice and (C3H × AKR)F_1 hybrids and, in contrast to the findings of Gross, none of the extracts elicited parotid tumors. Tissue sources for some of these extracts were: parotid tumors, mammary tumors from C3H mice, normal C3H tissues, and normal AKR tissues. Thus, these investigators found parotid tumors in only those mice which had received material from tissue culture preparations, from a transplanted AK/n leukemia, or from a paraganglioma. Stewart *et al.* (1957b), during a study designed to ascertain whether the virus of lymphocytic choriomeningitis could be implicated in the production of leukemia, added cell-free extracts of a transplanted AK/n leukemia to tissue cultures of monkey kidney and to cultures of chick chorioallantoic membrane. When fluids from these cultures were injected into (C3H × AKR)F_1 hybrids, some of the test animals developed parotid tumors.

Law *et al.* (1955) noted the occurrence of parotid tumors in strain C3H mice, (C3H × AKR)F_1 hybrids, and (C3H × C3H/Fg)F_1 hybrids after they had received preparations from leukemic tissues. They made the significant observation that the tumors appeared in certain litters: of 55 C3H, 17 (C3H × AKR)F_1, and 10 (C3H × C3H/Fg)F_1 litters, the tumors were confined to 8, 5, and 3 litters, respectively. It is of interest that 10 of 28 (C3H × C3H/Fg)F_1 treated hybrids developed parotid tumors and, while none was observed in untreated C3H or AKR mice, 2 spontaneous cases occurred in untreated C3H/Fg mice. When filtrates from parotid tumors were administered to C3H mice or (C3H × AKR)F_1 hybrids, few parotid tumors occurred in the test mice, but a footnote contained information that transplants of an induced

tumor of the adrenal medulla was a "good source of parotid-gland tumor-inducing substance." In a later paper, Law (1957) recorded the occurrence of 6 spontaneous cases of parotid tumors in about 200 untreated C3H/Fg mice and, in one family of C3H mice obtained from Bittner, 5 spontaneous cases in a total of 70 mice. These latter tumors were in mice of the same substrain as those in which Gross found but one such spontaneous tumor in his total experience.

Perhaps Law's most important observation was the tendency of the parotid tumors to appear in certain litters, because there must be a reason for a distribution of this kind within an inbred strain. Law *et al.* (1955) procured 31 offspring from C3H females which had parotid tumors during the nursing period, but none of the offspring developed a similar tumor. It would be of much interest to know whether the parotid tumors in Gross' experiments were also confined to certain litters. Gross (1955b) stated that the first parotid tumor to appear in his mice was found on November 9, 1951, and within "a week or two" 2 additional mice in the same litter developed the same type of tumor.

Woolley and Small (1956) obtained parotid tumors in C3H mice which had been inoculated with centrifuged and filtered extracts of AK/n leukemias, but no mention was made of litter distribution. In a later paper, Woolley and Small (1957) included 11 parotid tumors appearing in C3H mice following administration of cell-free leukemic extracts, but failed to mention litter distribution, although they recorded that 4 mice bearing cortisone-induced parotid tumors were not litter mates. This paper is of particular significance because it contained a summation of Woolley's experiments in which he produced parotid tumors in mice exposed to chronic administration of cortisone. Of the high-leukemia strains used, strain AKR mice failed to develop parotid tumors, while 3.5 % of the C58 strain and 8.0 % of hybrids derived from C58 females and AKR males did so. Of the low-leukemia strains used, 9.3 % of the C3H Gross subline developed parotid tumors whereas none of the C3H Hummel subline (related to the NCI substrain) did so. They thought the results in these C3H mice suggested a substrain difference in response to cortisone. Of much more importance to the subject under discussion is the opportunity it gives investigators to test for the presence of agents in the parotid tumors. These tumors, those arising spontaneously in Law's mice, and the one described by Gross are the only parotid tumors found, thus far, in strain C3H mice which had not received materials from tissue extracts, and it is of utmost importance that cell-free extracts from such growths be examined for tumor-inducing agents. This could be an excellent approach to the problem of the relationship between the leukemia and parotid tumor viruses.

Dulaney (1956) reported that cell-free extracts prepared from AKR or AK/n leukemias produced parotid tumors in strain AKR mice and that the

mice with tumors were limited to 6 of 17 litters. These were the first tumors of this type to be produced in AKR mice and Dulaney's results, together with those of Woolley and Small (1957) suggest that the induction of parotid tumors in an inbred mouse strain may depend upon the kind of treatment to which the animals are exposed.

Dulaney *et al.* (1957) recorded the occurrence of parotid tumors in C3H mice of different substrains after they had received cell-free leukemia extracts. These tumors arose in 7 of 152 mice of the NCI substrain and in 5 of 45 animals of the Bittner substrain. No direct statement was made concerning the limitations of parotid tumors to certain litters but the occurrence of 5 parotid tumors in a litter of 5 mice was mentioned. Extracts of leukemias from AK/n mice inoculated by Gross or from AK/n mice bearing transplants of this tumor were more effective in producing leukemia, parotid tumors, and sarcomas than those prepared from spontaneous or transplanted leukemias in AK/n or AKR mice raised in their own laboratory. They also included a statement that cell-free extracts from parotid tumors were used to carry the virus through two serial transplant generations. Continuation of this animal passage strain should yield a virus which will be available to other investigators.

3. *Sarcomas*

Gross (1955a) first mentioned the occurrence of sarcomas in his experimental animals in a footnote by stating: "Among mice which developed parotid gland tumors, several also developed subcutaneous fibrosarcomas." In a later paper (Gross, 1955c) more specific data were presented. Centrifuged or filtered extracts from induced leukemias, or induced parotid tumors in C3H mice, and centrifuged extracts from normal lactating mammary glands of C3H mice, were injected into C3H animals, of which some developed parotid tumors and sarcomas while others developed sarcomas only. Most of these sarcomas arose in subcutaneous tissues, but some were found in the muscles, in the muscular or perineural fascia, and in the uterus. Gross had never observed sarcomas in untreated C3H mice and, strangely, "only a few of these sarcomas could be transplanted, by cell transfer, in C3H mice of the same substrain." The tumors resembled fibrosarcomas, myxosarcomas, and rhabdomyosarcomas.

Gross (1956) prepared centrifuged extracts from induced parotid tumors in 2 C3H mice and used the material to inoculate 19 C3H mice; 5 developed spindle cell sarcomas at 12 -19 months of age. Sarcomas arose in other C3H mice treated with lyophilized and glycerinated AK/n leukemic tissues. Gross again emphasized the fact that he never saw a spontaneous sarcoma in his C3H mice. In his summarizing publication, Gross (1957b) showed that sarcomas were induced in C3H mice after they had been inoculated with

cell-free centrifuged or filtered material from AK/n spontaneous leukemias and with similar materials from induced leukemias, parotid tumors, and sarcomas in C3H mice. Thirty-four induced sarcomas were used to procure transplantable tumors. Cell suspensions were used to transplant 28 of these and only 10 were transplantable. When pieces of the 6 other primary tumors were used as inocula all were transplantable. This latter finding is in accord with almost all other recorded efforts in the literature when sarcomas arising in inbred C3H mice were transplanted into other members of their strain of origin. It would be of interest to know whether extracts prepared from these transplanted sarcomas, or sarcomas induced with a chemical carcinogen in C3H mice, would produce leukemia or parotid tumors in other C3H mice, because when Gross used materials from C3H sarcomas, induced with extracts of leukemic tissue, to inoculate 355 C3H mice, 26 developed leukemia, 19 developed parotid tumors, 13 developed subcutaneous sarcomas, and 157 mice were alive and tumor-free at a mean age of 11 months.

Woolley and Small (1956, 1957) confirmed the occurrence of sarcomas in the Bittner substrain of C3H and in Gross' line of the substrain following administration of extracts from AK/n leukemic tissues. They (Woolley and Small, 1956) supported Gross' claim that spontaneous sarcomas were very rare in C3H mice with the statement: "Parotid-gland tumors and sarcomas are virtually unknown in strain C3H." The writer is not aware of any published data on the occurrence of spontaneous sarcomas in the Bittner substrain other than the statement mentioned above, but it has been known for years that they do appear in the NCI substrain, and Dunn *et al.* (1956) and Heston (1958) have recorded such information. The spontaneous growths developed at a later average age than those appearing in mice treated when newborn with cell-free materials (Law, *et al.*, 1955). The spontaneous occurrence of these sarcomas does not imply they arose in the absence of a virus. As shown in the discussion of mammary tumors in this chapter, high or low incidences of this tumor may be procured in mice carrying or free of the mammary cancer virus. Hence, a high or low incidence of any tumor within an inbred mouse strain is no reason for assuming that the animals harbor or are free of a tumor virus. Strain C3H mice are highly susceptible to the development of sarcomas at the site of inoculation of carcinogenic hydrocarbons and these tumors should be used to ascertain whether the mice carry a sarcoma virus.

Dulaney *et al.* (1957) found 10 sarcomas in 152 mice of the NCI substrain of C3H following inoculation with AK/n leukemia extracts and stated that they arose "chiefly in very old animals." Law *et al.* (1955) obtained 9 subcutaneous sarcomas in hybrids inoculated with extracts of leukemic tissues; 8 arose in $(C3H \times C3H/Fg)F_1$ hybrids, of which 7 also had parotid tumors, and 1 arose in a $(C3H \times AKR)F_1$ hybrid with parotid tumors. Two subcutaneous

tumors in another (C3H × C3H/Fg)F_1 mouse were of interest because they displayed the histological morphology of parotid gland tumors.

4. *Adrenal Tumors*

These tumors, of medullary origin, were found by Stewart (1955a) in C3H mice inoculated with extracts of filtrates from a transplanted leukemia originating in a C3H mouse that had received, into the anterior chamber of the eye, a fetal thymic transplant which had been exposed to an extract of an AKR transplanted leukemia. Of 25 inoculated mice, 9 developed adrenal tumors, of which 7 were bilateral. The variety of other tumors in mice with adrenal tumors was of interest: 3 had adrenal tumors only; 2 had parotid tumors; 1 had a subcutaneous sarcoma; 1 had an ovarian tumor of the same cell morphology as the adrenal tumor; 1 had a similar ovarian tumor, a parotid tumor, and a mammary tumor; 1 had a parotid tumor, a mammary tumor, and a Harderian-gland tumor. Some of the adrenal tumors metastasized and subcutaneous transplants killed their hosts in from 8 to 12 weeks. Five other adrenal tumors were included in a later report by Stewart (1955b). These all rose in (C3H × AKR)F_1 hybrid mice, of which 4 had received filtrates from a transplanted AK/n leukemia and, of these 4 animals, 3 had mammary tumors and parotid tumors and 1 an adrenal tumor only. Stewart *et al.* (1957a) reported the occurrence of adrenal tumors in 6 (C3H × AKR)F_1 hybrids after they had received supernatant fluids from tissue cultures. These mice had also developed parotid tumors. The multiplicity of other tumors in mice with adrenal tumors could be significant.

Law *et al.* (1955) confirmed the occurrence of adrenal tumors in C3H mice that had received leukemic materials. Two mice developed tumors after receiving materials from AKR leukemic tissues, and both had parotid tumors. One tumor was transplanted and the workers found it to be a good source of materials for the production of parotid tumors. Stewart *et al.* (1957a) used cell-free extracts of adrenal tumors to inoculate 35 strains C3H and 26 (C3H × AKR)F_1 hybrids of which only one C3H mouse developed a tumor and this was an adrenal tumor. Gross (1957b) also observed the occurrence of adrenal tumors in his experimental animals.

5. *Discussion*

These studies of mouse leukemia permit few definite conclusions. This state of affairs is to be expected, not only because of the short period of elapsed time since the first observations were published, but because the problem has become involved in a complex of different tumor types. Gross (1957b) has assembled impressive evidence that cell-free extracts of leukemic tissues from AK/n mice contain an agent which promotes the occurrence of

leukemia in the Bittner substrain of C3H mice. In over 300 of these mice used as test animals, 28 % developed leukemia, and extracts from these leukemias resulted in the appearance of the disease in 37 % of 175 mice. It is regrettable that the only available incidence of spontaneous leukemia for this substrain comes from the colony maintained by Gross, but it is very doubtful that the normal incidence approaches 28 37 %. Gross' evidence, supported by the findings of Woolley and Small (1957), indicates that their procedures did elicit leukemia in the C3H mice. Oncologists familiar with inbred mice strains used in cancer research are not too much concerned over the conflicting results obtained when the NCI and Bittner substrains were used to investigate the leukemia virus It has been known for many years that these substrains differ in their tumor responses as well as in other ways. Further evidence of a leukemia virus was presented by Gross when he showed that, in his hands, cell-free extracts of AK/n leukemic tissues produced the disease in C57BR/cd mice and similar extracts from strain C58 leukemic tissues did so in strains C3H and C57BR/cd. Finally, his recent efforts to acquire a transmissible strain of the virus are most encouraging, for it may now be possible to characterize its physical, chemical, and biological properties and thus compare it with other known tumor viruses.

Much remains to be done. The activity of the virus in newborn mice of other strains and, of more importance, further studies of the age factor in resistance to it must be pursued. Serological and tissue culture studies are essential to keep apace with modern techniques for investigation of a virus.

Perhaps one of the major handicaps investigators of this problem have imposed upon themselves is their tendency to focus attention upon their immediate problems and their failure to interpret their efforts in relation to the broad problem of mouse leukemia. Dunn (1954) has written an excellent review of the disease in mice and has presented a classification of its numerous forms which should serve as a meeting ground for those working in the field to assure themselves that they are working with the same type of lesion. Gross' (1957b) review, for example, contains no reference to Dunn's paper or to those of other pathologists who have studied the morphological forms of the disease. It should also be kept in mind that it has also received attention from excellent investigators who have done much to establish the importance of hereditary factors, chemical and physical carcinogenic agents, hormones, and the role of the thymus in the occurrence of the disease. Law (1957) has written a concise review of these efforts which contains most of the pertinent references. The lack of effort on the part of recent workers to discuss the applicability of their findings to the known factors involved in the occurrence of mouse leukemia is disappointing. The thymus gland is recognized as the primary site of origin of the disease in the AKR and C58 mouse strains; if the thymus is removed from these mice, their tendency to develop spontaneous

leukemia is greatly reduced. Can the agent be detected in the thymus before the occurrence of the disease, in the same manner that the virus of mouse mammary cancer is detectable in the milk? What influence will thymectomy exert upon the production of the disease by the inoculation of filtrates from leukemic tissue extracts? Is the agent in tissues of low-leukemia strains of inbred mice developing the disease after exposure to chemical carcinogens, ionizing radiations, or hormones? It is conceivable that all cases of leukemia in mice are not caused by a virus. Here, experience gained with the mammary tumor virus of mice could be of some assistance to those interested in leukemia. The scope of the problem with this tumor virus is so large and the practical implications so important that researchers engaged in the work are obliged to utilize every assistance offered by their predecessors, and, more important, to co-operate fully with their contemporaries

All who have administered extracts of leukemic tissues to newborn mice agree their test animals developed parotid gland tumors, and it would now appear that investigations of this tumor are providing more interest than the leukemia studies. Mice of the NCI and Bittner substrain of C3H mice develop parotid tumors after receiving leukemic extracts but, thus far, spontaneous tumors of this type have been observed only in the Bittner substrain. If mice develop this tumor because they harbor the virus, then it must be in both substrains. But when Gross (1957b) used cell-free extracts from C3H parotid tumors as inocula for other C3H mice, only 7 % responded with similar tumors and Stewart *et al.* (1957a) reported negative results in their C3H mice after receiving similar extracts. Dulaney *et al.* (1957) casually remark they have carried the agent through 2 serial passages by means of filtrates. When Gross (1957b) used extracts of parotid tumors as inocula for C3H mice, he observed a leukemia incidence of 16 % in the test animals, but when Stewart *et al.* (1957a) used tissue cultures infected with extracts of parotid tumors or leukemic tissues as inocula for (C3H $\times$ AKR)F_1 hybrids, none developed leukemia. The variety of tumors and other lesions in Stewart's mice, and their occurrence only in animals with parotid tumors, raises the question whether the other tumors would arise in mice whose parotid glands had been removed.

Schmidt (1956) injected infant mice with a fraction from the cytoplasm of cells of the transplantable Ehrlich carcinoma and observed the appearance of malignant tumors of different types, including salivary gland tumors, in the inoculated mice. It is obvious that the problems of this agent require clarification.

The appearance of leukemia, parotid tumors, and sarcomas in his test mice posed a major problem for Gross. He discussed frequently the possibility of the same virus or different viruses being responsible for the various lesions and, while inclined to favor the view that different agents were responsible,

closed his discussion of the most recent paper reviewed here (Gross, 1957b) with the words, "It would not be surprising to find that such agents, although possibly of a different pathogenic potential, were related." Dulaney *et al.* (1957) were not so cautious and stated: "It is our belief that the parotid gland tumor-inciting agent is separate and distinct from the leukemic agent."

The problem became more complicated when Stewart *et al.* (1957a) reported the occurrence of a wider variety of tumors in mice receiving tissue culture preparations or cell-free extracts from a transplanted AK/n leukemia. In addition, Eddy *et al.* (1958) found that the tissue culture materials elicited different tumor types in newborn hamsters. The implications of this work with tissue cultures are far reaching and emphasize again the application of tissue culture techniques to the tumor viruses.

The development of the problem of a single virus or multiple viruses being causative agents of these tumors will be watched with interest, especially by those who are working with the chicken tumor viruses. It will be recalled these investigators have already advanced the unitarian theory of tumor viruses and the concept of a stem virus without the benefits derived from the use of inbred animals as experimental animals. The establishment of high-leukemia strains of mice has been of invaluable assistance to those now working in the field, and, perhaps, they should reciprocate by giving more attention to the host. It should be firmly established that only newborn mice are susceptible to the agents because, if substantiated, it introduces a new concept into the problems of the tumor viruses. A recent report by Gross (1958) revealed that filtrates of a potent "passage" strain of leukemia induced leukemia in the Bittner substrain of C3H mice which were from 1 to 51 days of age. Cell-free extracts could be administered to newborn and older mice of a variety of inbred strains to establish a firm foundation for future work. Such efforts will not only assist the progress of the immediate problem, but will contribute to cancer research by providing information of the value and the limitations of the newborn as hosts for tumor viruses. It is known that newborn animals are in a stage of immunological nonreactivity and this demands a thorough investigation to ascertain the importance of the hosts in all investigations reviewed here.

Space does not permit a satisfactory review of the efforts of others who have worked with leukemia agents. Friend (1957a,b) discovered an agent in an Ehrlich ascites tumor which has been transferred serially through noninbred adult Swiss mice by means of cell-free filtrates. Whether the virus induces a disease belonging to the categories described by Dunn (1954), or whether it is a variant of the mouse leukemia virus, comparable to the viruses of erythroblastosis or myeloblastosis in their relationship to the virus of visceral lymphomatosis of chickens, is the major problem.

Schwartz *et al.* (1956) prepared filtrates from the brains of high-leukemia strain AKR mice with spontaneous, transplantable, or induced leukemias and injected the filtrates into other AKR mice which were over 4 weeks old. The inoculated animals were killed when 22 weeks of age, when between 45 and 100 % had developed leukemia. Of 229 AKR mice inoculated with various control materials, none showed evidence of a similar acceleration of the leukemic process. Engelbreth-Holm and Frederiksen (1938) and Rudali *et al.* (1956) also observed an acceleration in the appearance of leukemia in strain AKR mice after they got cell-free extracts of AKR tissues. In a later paper Schwartz *et al.* (1957) reported, with similar procedures and the use of 3-to 16-week-old AKR mice as test animals, the early occurrence of leukemia following injections of filtrates prepared from the brains of patients who had died of acute leukemia. Control materials, consisting of heat-treated filtrates from the same patients, did not hasten the appearance of leukemia in AKR mice. It is regrettable that a few mice from a low-leukemia strain were not used along with those of the AKR strain.

In another communication from this group of workers (Schoolman *et al.* 1957) mice over 4 weeks of age were used. Briefly, a spontaneous leukemia arising in a Swiss mouse (apparently noninbred) was transplantable, to a limited extent, in other Swiss and inbred DBA mice. Filtrates from these Swiss or DBA tumors failed to produce tumors in Swiss mice, whereas filtrates from brains of tumor-bearing Swiss or DBA mice evoked leukemia in Swiss or DBA mice. Strangely, filtrates from leukemic brains of either strain failed to accelerate the occurrence of leukemia in AKR mice. The investigators stated the crux of their problem as follows: "The nature of the agent in these filtrates has not been defined," but the closing statements in their paper give the impression it was transmissible in serial passage.

Graffi (1957) has recently summarized the work of another group of investigators interested in problems of mouse leukemia. Space does not permit a thorough discussion of this review, but these workers have detected agents in 5 transplantable mouse tumors which, when administered to test animals, induced myeloid leukemias, "chloroleukemia," instead of the usual lymphatic leukemias obtained by others. Newborn mice and adults were used as recipients for tumor filtrates, but the review contained few references to the use of adults. Mice from birth to 11 days of age were equally susceptible to filtrates from one sarcoma and even mice 3 to 4 months old were "rather" susceptible.

Other interesting findings were: (1) No chloroleukemias were found in mice treated with embryonic tissues from mice, rats, or chickens. (2) Tissue cultures of mouse embryos were inoculated with tumor filtrates, and, after one or more passages, were filtered and the filtrates injected into newborn mice. The results were negative, but the inocula were "very toxic." (3) The agent in

filtrates from one tumor was neutralized by rabbit antisera but not by mouse antisera. (4) Despite the 100 % transplantability of the original tumors, the filtrate-produced leukemias grew in only 10 % of the hosts and serial transplantation was successful in only four instances. This was an unusual finding, especially when "inbred strains" were used as hosts.

The chief contribution studies on the viral etiology of mouse leukemia have made to the tumor viruses is to arouse the active interest of many oncologists and virologists. The work, thus far, has produced results of considerable significance, but so much is dependent upon future studies that any further conclusions would be purely speculative.

III. Conclusion

This chapter was written for readers who are interested primarily in viruses and secondarily in an objective review of the role of these agents in the causation of cancer. The virus theory of cancer requires no defense. The best summation of experimental evidence in support of the theory was presented by Rous (1936) more than twenty years ago, and the best reason for its use in the experimental approach to the cancer problem was given by Andrewes (1950) who found it "intellectually satisfying." Any concept based upon sound scientific data and capable of stimulating further research is certain to contribute much to knowledge of the cancer process.

The crux of the problems of the tumor viruses is to learn more of the properties of the known viruses and to expose others. Perhaps the chief lesson to be learned from past experience is that almost all rewarding efforts with the tumor viruses have come from working directly with tumors or with the known tumor viruses. Virologists interested in cancer research should be alert to advances in the entire field of virology and to the application of newer knowledge to their problems. But efforts to establish a viral causation of cancer by invoking analogies between the known characteristics of other viruses and the cancer process would appear to be futile.

This chapter may have been written at the poorest possible time. The problems of the tumor viruses are being advanced so rapidly through excellent investigations of the known viruses, the concentration of effort upon the problem of mouse leukemia, and the convergence of virus research and tissue culture research, that within a few years definitive answers may be had to the viral causation of many other cancers.

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Chapter XIII

The Insect Viruses

KENNETH M. SMITH

Agricultural Research Council, Virus Research Unit, Cambridge, England

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I. INTRODUCTION

The early work on the viruses affecting insects was devoted exclusively to the polyhedral virus diseases, so called because of the presence in certain tissues of polyhedra, the many-sided crystalline inclusions associated with this type of virus. The reason for this exclusive study is obvious enough since the polyhedra are easily visible on the optical microscope, thus making diagnosis easy. Although intensive study of the insect viruses is only of recent origin, we know that there are at least three types and of these, the polyhedral viruses are divisible into two quite distinct groups.

The kinds of insect viruses, then, which are known so far, are the polyhedral virus diseases or *polyhedroses;* these consist of the nuclear and cytoplasmic diseases according to the site of virus multiplication. Secondly, there are the granular diseases, or *granuloses*, in which the intracellular inclusions are extremely small crystals—the granules from which the disease takes its name. Thirdly, there are those diseases in which no intracellular inclusions have been observed.

Several hundred virus diseases of the polyhedral type have been recorded and about thirty or so granuloses. The number of viruses without accompanying intracellular inclusions, so far described, is small but this is probably because of the greater difficulty involved in their diagnosis. There is little doubt that many more insect viruses have yet to be described.

The distribution of the known viruses in the insect kingdom is very uneven and this is probably more a reflection of our lack of knowledge than a true state of affairs. However this may be, viruses are only known for certain to affect the Lepidoptera, Hymenoptera, and Diptera. None have so far been reported from the Hemiptera which includes the aphids and plant bugs, the Orthoptera containing the grasshoppers and locusts, or the Coleoptera with its forty thousand species of beetles, though a rickettsia-like organism has recently been described in *Melolontha* sp. (Krieg, 1955).

It is an interesting fact that in almost every case it is the larval stage only of the insect which is susceptible to infection. Occasionally, caterpillars may pick up the virus in a late instar and complete their development as far as the pupal stage, but the pupae die of the disease. Very rarely the adults may become infected. On one occasion in this laboratory a number of larvae of the privet hawk moth, *Sphinx ligustri*, were infected with a nuclear polyhedrosis. The majority died but a few survived sufficiently long to pupate; of the resulting pupae one female moth emerged but died shortly after. The tissues of this moth were found to be filled with polyhedra.

There seems to be only one case where the adult form of an insect is susceptible to a virus but not the larva; this is the so-called bee paralysis where the adult bee is attacked (Burnside, 1933).

As we shall see later, there is a good deal of variation in the morphology of insect viruses, as is the case with the viruses affecting plants and higher animals, but within each group the morphology is fairly constant. It is interesting that, up-to-date, the viruses which develop in the cell nuclei appear to be all rod-shaped, while those multiplying in the cytoplasm are spherical or near spherical.

Between them the various types of viruses seem to attack practically all the tissues of the larval insect, and polyhedra have been observed in the skin, tracheae, fat body, blood cells, gut, gonads, muscles, imaginal wing and limb buds, nerve ganglia, Malpighian tubules, and silk glands.

II. Different Types of Insect Viruses and the Diseases Caused

A. Polyhedral Viruses

1. *Nuclear Viruses*

Most of the early work on polyhedral diseases was carried out on the so-called jaundice of *Bombyx mori*, the silkworm, which is the classic nuclear

polyhedrosis. According to Steinhaus (1949), Cornalia (1856) and Maestri (1856) were among the first to observe the polyhedra and associate their presence with the disease. Following this a long controversy ensued as to the nature of the polyhedral bodies, various suggestions being put forward, the most popular being that the polyhedra were some kind of organism. In 1907, von Prowazek showed that material from diseased silkworms was still infectious after removal of the polyhedra by passage of several layers of filter paper. This was the first step towards the idea that silkworm jaundice was due to a filterable virus; further proof was forthcoming through the work of Acqua (1918-1919) and Paillot (1924, 1926a,b, 1930). Using dark-ground illumination, Paillot saw numbers of minute granules which he believed to be the agent causing silkworm jaundice. Komárek and Breindl (1924) also showed that minute bodies inside the polyhedra could be seen with the optical microscope. This was confirmed by Bergold (1947), who dissolved the polyhedra with weak alkali and observed the liberated virus rods on the electron microscope. It is now known that the polyhedra are protein crystals with the virus particles as an occluded constituent. Without the virus particles the polyhedra are noninfectious.

The infectivity of the virus rods from the nuclear polyhedra was shown by Bergold (1947) and Bergold and Friedrich-Freksa (1947). They established three facts: (1) The infectivity of the isolated particles is 1×10^{-11} to 4×10^{-11} gm. per larva; this is several orders higher than that of a polyhedral solution before concentration. (2) The sedimentation constant of a suspension of virus particles calculated by infectivity tests of different sedimentation levels is in good agreement with that obtained by the usual optical method. (3) Serological investigations show that the isolated virus particles are serologically unrelated to the host serum and only slightly related to the low molecular weight polyhedral protein.

The nuclear polyhedra do not stain with Giemsa solution or iron hematoxylin without some previous treatment with acids or alkalis; this was first shown by Escherich and Miyajima (1911). The staining reaction is an important one for differentiating between the nuclear and cytoplasmic polyhedra, since, as we shall see subsequently, the latter stain readily with Giemsa solution without any treatment.

Aqueous or alcoholic solutions of bromophenol blue, with or without mercuric chloride, do not stain nuclear polyhedra in spite of their highly proteinaceous character. However, after 10 minutes of pretreatment in normal hydrochloric acid at 60°C., the polyhedra stain intensely with bromophenol blue, with or without mercuric chloride. After the same pretreatment the polyhedra stain pink with dilute Giemsa solution.

Although it is possible to make out the bundles of virus rods inside the polyhedra in unstained preparations, by means of the optical microscope,

they are rendered more visible by the following technique: Sections of diseased larvae, fixed in Carnoy's solution, are treated with I *N* HCl at 60°C. for 10 minutes, stained in diluted Giemsa solution for 20 hours, and differentiated in 95 % alcohol for 5–20 minutes. Each virus bundle within and without the polyhedra stains a deep reddish-purple and the polyhedral protein stains a weak pink color. The distribution of the virus bundles within the smallest and largest polyhedra can be clearly seen (Xeros, 1953a).

a. Location of Nuclear Polyhedra in Insect Tissues. It is becoming fairly well established that practically all the larval tissues are susceptible to infection with the nuclear polyhedral viruses. Breindl (1938), however, considered that the gonads, the Malpighian tubules, and the alimentary tract were immune to infection. More recent work has shown this view to be erroneous and in a nuclear polyhedrosis of the clothes moth larva, *Tineola bisselliella*, typical polyhedra have been found in all the following tissues: hypodermis, fat body, silk gland, Malpighian tubules, nerve ganglia cells, imaginal buds, fore-gut, and hind-gut (Smith and Xeros, 1954a). The occurrence of nuclear polyhedra in the cells of the gut is rather unusual but, as we shall see later, the gut (especially the mid-gut) is the main site of multiplication of the cytoplasmic polyhedral viruses.

It has also been shown that in the case of a nuclear polyhedrosis of the European pine sawfly, *Neodiprion sertifer* (Geoffr.), polyhedra were found only in the nuclei of the digestive cells of the mid-gut epithelium (Bird and Whalen, 1953).

Perhaps the most usual tissues infected with the nuclear polyhedroses are the hypodermis, tracheae, fat, and blood cells. The fact that the skin is so frequently attacked is the underlying cause of the most characteristic disease symptom. In these diseases and in the granuloses, where the skin is also attacked, the affected larva becomes a limp sac with a fragile integument which ruptures at a touch, liberating the semi-liquid body contents containing millions of polyhedra (Fig. 1). The bodies of larvae which have died of a nuclear polyhedrosis frequently hang in a characteristic manner from the food plant, attached by the middle abdominal feet, thus forming a kind of inverted "V". The habit of some infected larvae, particularly those of *Lymantria dispar*, of climbing to the top of the tree on which they have been feeding, and then hang down in the manner described, has earned in Germany the name of "Wipfelkrankheit," or tree top disease.

b. Development of Nuclear Polyhedra. The intranuclear changes in infected cells vary considerably in different species (Xeros, 1953a), but the main features of the sequence of intranuclear changes are identical. The nucleus of an infected cell grows enormously; this is particularly true in the nuclear polyhedrosis of the dipterous larva, *Tipula paludosa*, in which the nucleus enlarges almost to the confines of the blood cell (Fig. 2). The chromatic

material, which is greater in bulk than that of normal nuclei, clumps together to form an off-central mass; the remainder becomes attached to the inner surface of the nuclear membrane, to which the central chromatic mass is also apparently attached. Between the two lots of chromatic material a clear ring zone develops, refractive to staining. Later, after the transformation of the central chromatic mass to a network, polyhedra form in the ring zone and grow from about 0.2 to 0.3 to 1 μ or more in diameter, and may even reach 10 μ. In the cases of *Lymantria dispar* and *L. monacha*, large numbers of virus bundles are accumulated in the ring zone and, as the polyhedra grow, the virus becomes embedded in them. In these two species the polyhedra often originate by deposition of polyhedral protein around aggregations of large numbers of virus particles. As the growing polyhedra begin to pack the nuclei, more and more virus bundles are found inside them and fewer and fewer outside them.

In infected nuclei of *Bombyx mori* the course of intranuclear events is somewhat different. In this species, visible virus bundles are not often found in the ring zone and electron microscopy of the virus contents of the polyhedra confirms that the virus rods in this species are almost exclusively in the form of single virus rods. Moreover, immediately prior to the formation of polyhedra, considerable and increasing amounts of protein material, staining a pale pink with Giemsa and a medium blue with bromophenol blue, is found precipitated in the ring zone. Later, relatively few and very large polyhedra form in the infected nuclei from this protein and leave the nuclear sap free of visible protein deposit. In this species, then, large amounts of polyhedral protein are to be found in the ring zone even before polyhedral formation (Smith and Xeros, 1954a).

By means of bromophenol blue staining technique, Xeros (1953b) has studied the polyhedra formation in more detail. At about the time of the transformation of the chromatin mass to a network, a great number of propolyhedra appear for the first time in the nucleus. They first appear outside the chromatic network and not as a rule in its pores; the smallest of the propolyhedra observed are about 0.2–0.3 μ in diameter, and are found around the network and at the periphery of the nucleus. These grow into mature polyhedra, 1.5 μ or more in diameter, by which time they pack the nucleus. As the polyhedra grow and begin to pack the nucleus, the nuclear net undergoes further changes. It may expand to embrace a much greater volume of the nucleus and enclose, secondarily, many polyhedra in its enlarged pores.

Hughes (1953) has studied polyhedral formation in a nuclear disease of the alfalfa caterpillar, *Colias philodice eurytheme* Bdl. He considers that the polyhedral bodies start as bundles of virus particles enclosed in globular membranes. An elaboration or deposition of some dense material within these

membranes results in the whole structure becoming opaque to the electron beam. Then, as several of these units occur in close proximity, a further deposition of dense material around and between them embeds a number of them in one mass.

In nuclear polyhedroses of two hymenopterous insects, *Diprion hercyniae* (Htg.) and *Neodiprion americanus banksianae* Roh., polyhedron formation commences in the chromatin. The chromatin of *D. hercyniae* often coagulates in such a manner that separate lumps suggest small polyhedra; the lumps then transform into recognizable polyhedra. The chromatin of *N. americanus banksianae* is more uniformly dispersed, and polyhedra arise as thickenings within the chromatin. In both insects, the polyhedra are found in ever-increasing numbers in the nuclear sap, where they are larger and denser than those in the chromatin (Bird and Whalen, 1954).

In the nuclear polyhedrosis of *Tipula paludosa*, a chromatic mass forms in the enlarged nuclei of the blood cells, and from this mass the chromatic material segregates as several spherical bodies; the polyhedra seem to arise around the periphery and are closely applied to the nuclear membrane. They are negatively birefringent and appear to be genuine crystals (Smith and Xeros, 1954b).

c. Different Shapes and Sizes of Polyhedra. The nuclear polyhedra vary greatly in shape and size and may be dodecahedra, tetrahedra, cubes, or, in the case of a polyhedrosis of *Tipula paludosa*, cresent-shaped. As a rule the polyhedra in one nucleus are of the same size but the size varies in different nuclei and as many as 100 or more may occur in the same nucleus (Fig. 3). Very often a particular polyhedral shape is characteristic for a particular host species, e.g., cubic in *Panaxia dominula* and crescent-shaped in *T. paludosa.*

The great variation in shape and size of polyhedra may give rise to some difficulty in diagnosis under the optical microscope. The fact that sometimes the polyhedra appear almost spherical tends to confuse then with uric acid crystals and pupal bodies, etc. However, after some practice in the recognition of the various types of polyhedra, there is usually not much difficulty in making a correct diagnosis from a smear stained with Giemsa solution. Krieg (1957) describes methods for physical and chemical differentiation between genuine polyhedra and the various other rather similar entities.

d. Dispersal and Arrangement of Virus Rods inside Polyhedra. Bergold (1947) calculated that the polyhedral bodies consist of about 95 % by weight of noninfectious protein and about 5 % of infectious virus particles. This is probably only approximate, for there seem to be great variations in the amount of virus contained in the polyhedra of different nuclear polyhedroses and even in individual polyhedra of the same disease. The number of virus rods in the polyhedra from *Lymantria dispar* and *Bombyx mori* is sometimes very large,

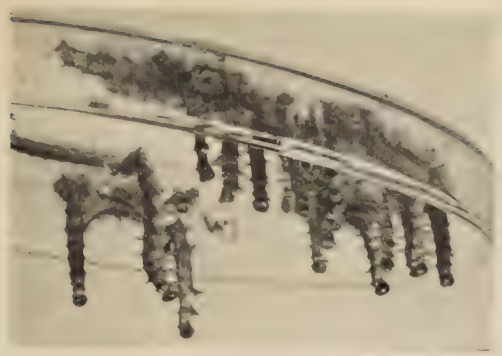


FIG. 1. Larvae of *Aglais urticae*, the small tortoise-shell butterfly, killed by a nuclear polyhedrosis. Note the characteristic position of the dead caterpillars. (Magnification: × 1.)

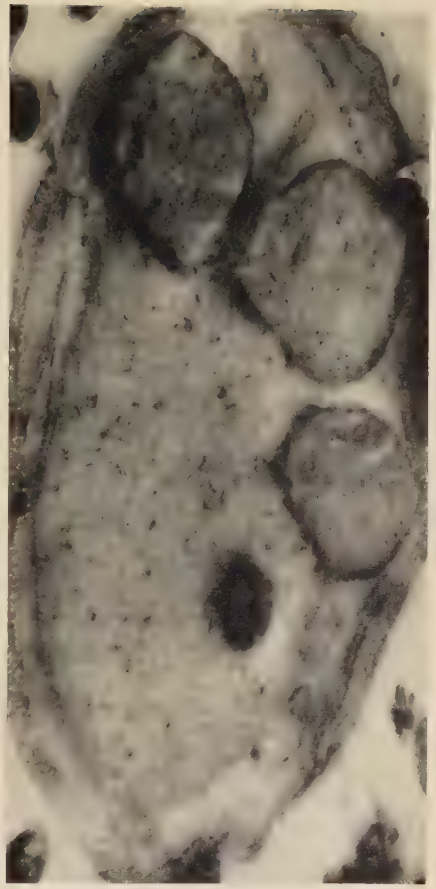


FIG. 2. Thin section of a blood cell from a larva of *Tipula paludosa*, the crane fly, infected with its nuclear polyhedrosis. Note the greatly enlarged nucleus with its double membrane and three polyhedra containing the rod-shaped virus particles. (Magnification: × 17,000.)

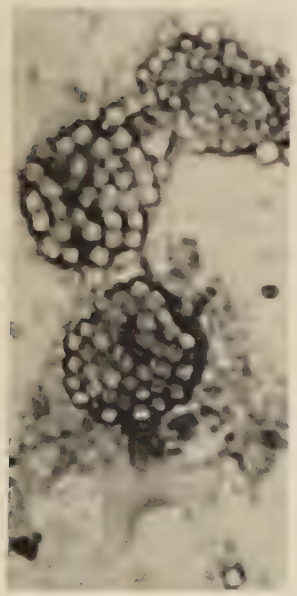


FIG. 3. Blood cells from a larva of *Sphinx ligustri* infected with a nuclear polyhedrosis. Note the large numbers of polyhedra. (Magnification: × 530.)

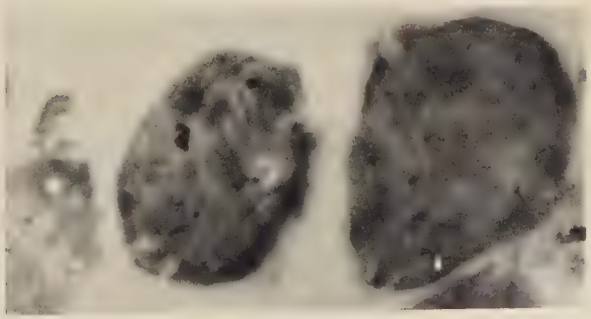


FIG. 4. Thin section of two nuclear polyhedra from *Euvanessa antiopa*. Note the haphazard arrangement of the occluded virus rods. (Magnification: × 30,000.)

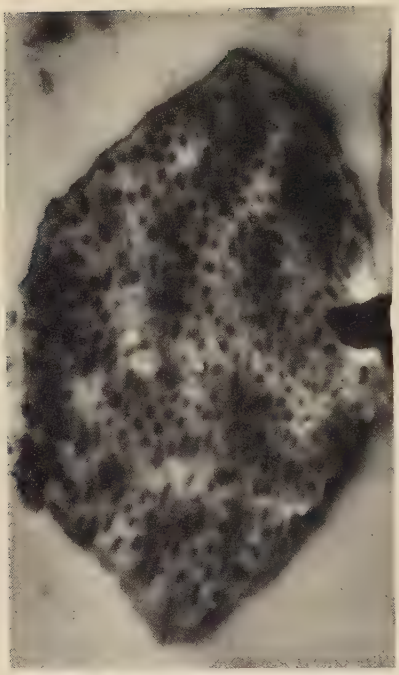


FIG. 5. Thin section of a nuclear polyhedron from a larva of *T. paludosa*; there is some suggestion of a regular arrangement of the virus rods. (Magnification: × 30,000.)

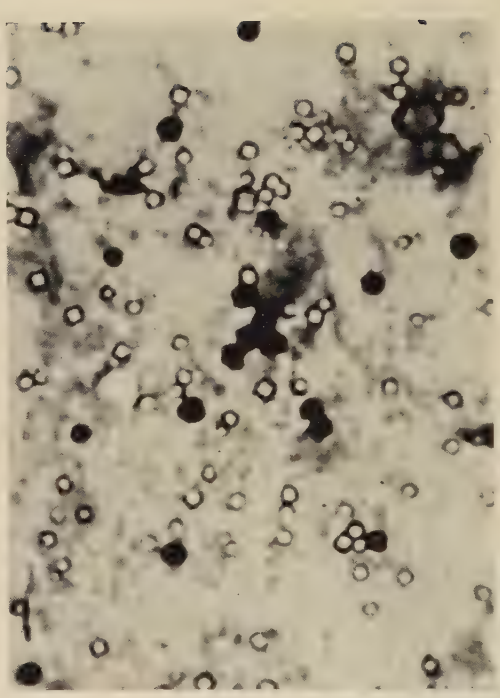
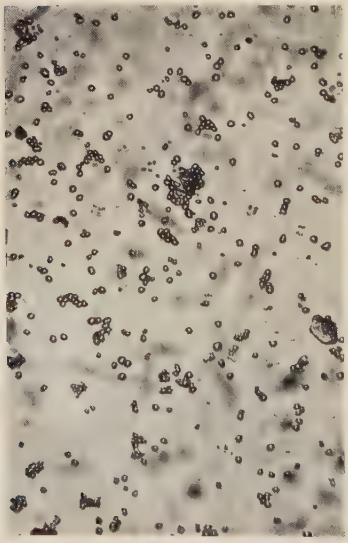


FIG. 7. Blood smear from a larva of *Sphinx ligustri* infected with a nuclear and a cytoplasmic polyhedrosis. Note the stained crystals of the latter. (Magnification: × 630.)

FIG. 1, 3, 6 and 7 from Smith and Xeros, 1953b, 1954 a,b,c.

(a)



(b)

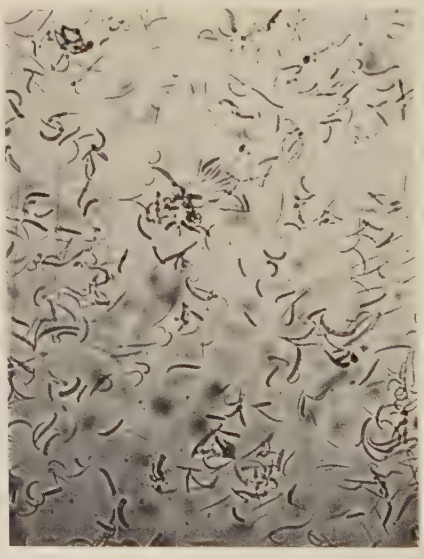


FIG. 6. (a) Nuclear polyhedra with *T. paludosa* before treatment from weak alkali; (b) the same polyhedra after treatment. Note the peculiar elongation of the crystals. (Magnification: × 150.)

while the number is much reduced in the polyhedra of *Tineola bisselliella* and *Euvanessa antiopa*.

There seems little doubt that in the nuclear polyhedra of the Lepidoptera the arrangement of the virus rods within the polyhedron is quite haphazard. This can be demonstrated by thin sections (Morgan *et al.*, 1956) (Fig. 4).

In the case of the nuclear polyhedrosis of a fly larva (*Tipula paludosa*), however, there is some suggestion of a more regular arrangement of the particles. Sections through the polyhedra (Fig. 5) give this impression and the striations visible in the polyhedra on the optical microscope may be an expression of the inner regular array of particles. On several occasions sections through the polyhedra of *T. paludosa* have revealed the virus particles lined up along the edge of the crystal; the significance of this is not very clear unless it is that the particles failed to become incorporated in the polyhedral body at the time of crystallization.

e. The Reaction of Nuclear Polyhedra to Alkalies, etc. Bolle (1898) was the first to show that the polyhedra dissolved in acids and alkalis and Paillot and Gratia (1939) observed the dissolution of polyhedra with weak alkali under dark-ground illumination. Bergold (1953) has worked out a number of concentrations of Na_2Co_3 for the dissolution of nuclear polyhedra from different species under standard conditions.

Polyhedra from different species differ greatly in their resistance to alkaline treatment, but the majority are dissolved by treatment for 5 minutes with 4 % Na_2Co_3. However, the polyhedra from larvae of *Pterolocera amplicornis* Walker withstand this concentration for 30 minutes and a treatment of at least 60 minutes at 56°C. is necessary to dissolve them completely (Day *et al.*, 1953).

The nuclear polyhedra from the dipterous insect *Tipula paludosa* are in a class apart from all other nuclear polyhedra so far described. They are resistant to trypsin and to dilute and weak acids and alkalis. In 1 *N* sodium hydroxide they elongate to six or more times their length, becoming first biconvex spindles and then elongating into crescents or wormlike shapes (Fig. 6). At about three times their normal length this elongation is still completely reversible and in water, at pH 5–8, they return to their normal shape and size. After such treatment, however, the polyhedra are "activated"; in other words, they now respond in a similar manner in ammonia, 1–12 % sodium carbonate, and hydrochloric acid, pH 1–4, but not to 1 *N* hydrochloric acid or 25 % sodium carbonate. The elongation and contraction or return to normal shape, which take place along the same axis, can be repeated indefinitely in these solutions and take place as rapidly as the solutions can be alternated (Smith and Xeros, 1954b).

2. *Cytoplasmic Viruses*

The existence of a separate and distinct type of polyhedral virus, which was spherical instead of rod-shaped, was first demonstrated by Smith and Wyckoff

(1950), and the differential staining properties of the polyhedra later investigated (Smith *et al.*, 1953). Previously, several workers had observed polyhedra in the cytoplasm of the mid-gut (Ishimori, 1934; Lotmar, 1941), but the fact that they were of an entirely different nature from the nuclear polyhedra was not realized.

The reaction of the cytoplasmic polyhedra to stains and alkalies differs sharply from that of the nuclear type and is an important aid in diagnosis. In smears made from infected caterpillars, fixed by mild heating and subsequently stained with methylene blue or Giemsa solution, the cytoplasmic polyhedra take up the stain readily. This is in marked contrast to the behavior of the nuclear polyhedra, which under these conditions do not stain at all. Figure 7 shows a smear made from a larva of the privet hawk moth, *Sphinx ligustri*, which had a double infection with both nuclear and cytoplasmic polyhedroses; note how the latter are clearly differentiated.

a. Location of Cytoplasmic Polyhedra in Insect Tissues. The number of cytoplasmic polyhedroses now recorded is extremely large and it seems highly probable that they are more numerous than the nuclear diseases; this, at all events, has been the writer's experience in his studies at Cambridge. In every case the virus appears to develop in the gut cells, usually the mid- or hind-gut, and in a late stage of the disease the cells become completely filled with polyhedra. These also occur in the lumen of the gut and are excreted in large numbers in the feces (Fig. 8).

In a cytoplasmic polyhedrosis of the silkworm, *Bombyx mori*, the polyhedra are produced in the cylindrical cells of the mid-gut epithelium but not in the goblet or interstitial cells (Tsujita, 1955). In a similar disease of the spruce budworm, *Choristoneura fumiferana* Clem., the polyhedra occur in the cytoplasm of the digestive cells of the mid-gut epithelium (Bird and Whalen, 1954).

The symptomatology of the cytoplasmic polyhedroses usually differs sharply from that of the nuclear disease. The main difference arises from the fact that the skin is not attacked and, in consequence, the larva, though limp and flaccid, does not disintegrate in the manner so characteristic of nuclear polyhedroses. In some species, the mid-gut is clearly differentiated through the skin by the accumulation of the polyhedra in the cells. This may occur quite early in the disease before the general health of the larva appears to be affected and is most marked in the cytoplasmic polyhedrosis of certain species, notably *Phlogophora meticulosa*, the large angleshades moth and *Diataraxia oleracea*, the tomato moth (Fig. 9).

b. Development of Cytoplasmic Polyhedra. Xeros (1957) has described the formation of the polyhedra in a cytoplasmic disease of the larva of *Thaumatopoea pityocampa*. Bodies which are apparently virogenic stromata appear in the epithelial cells before the formation of the polyhedra. Later, small

polyhedra form sparsely over the virogenic masses; these polyhedra vary in size but may be under 0.5 μ in diameter. They are best observed in relation to the cytoplasm and virogenic masses in HCl-Giemsa preparations. Further development proceeds by the continuing growth of the virogenic masses and by the increase in the number and size of the polyhedra at their surfaces. Polyhedron formation does not take place equally at all parts of these surfaces; some parts may be packed with well-developed polyhedra 0.5–1 μ in diameter, other parts may be completely free of them. During this stage of the disease, large pores develop within the virogenic masses and polyhedra also arise and develop within these pores. In the fully matured colonies of the moribund cell the polyhedra reach a size of 1.5 μ in diameter.

c. Different Shapes and Sizes of Cytoplasmic Polyhedra. On the whole, the cytoplasmic polyhedra resemble those of the nuclear diseases but there are differences. There seems to be a greater range of size of cytoplasmic polyhedra in an individual smear, from very small to very large indeed, sometimes as much as 10 μ. This was particularly the case in a cytoplasmic disease of *Ourapteryx sambucaria*. There is also a tendency on the part of the large polyhedra to lose their many-sided character and to appear almost spherical. In the spruce budworm, the polyhedra are chiefly cuboidal and triangular forms have not been observed (Bird and Whalen, 1954).

d. Dispersal and Arrangement of Virus Particles inside Polyhedra. It is the writer's opinion, after long experience of cutting thin sections of both types of polyhedra, that the cytoplasmic type is considerably harder and it is more difficult to get good sections without much compression by the knife. This gives the impression that the viruses are somewhat elongated but in fact this is only an artifact due to compression. Furthermore, some of the cytoplasmic viruses are very small, one affecting the larva of *Sphinx lingustri* measures 12mμ, about half the size of the tomato bushy stunt virus (Fig. 19), and it is difficult to observe them at all in sections. Indeed, from observation of the sections alone it would appear that little or no virus was present, whereas in actual fact the virus content is very high. Observation is rendered more difficult by the lack of contrast between the virus particle and the surrounding polyhedral protein (Fig. 10).

On the whole, it appears that the particles are arranged haphazardly within the polyhedral crystal. Occasionally, however, sections may reveal what appears to be a regularity of assembly within the crystal (Fig. 11).

e. Reaction of Cytoplasmic Polyhedra to Alkalies. It is in their response to treatment with weak sodium carbonate that the two types of polyhedra differ most. We have seen that the nuclear polyhedra dissolve completely, leaving behind a membrane in which the virus rods are contained (Fig. 12). With similar treatment the cytoplasmic polyhedra dissolve only partially and

a honeycomb or spongelike residue remains; there is no membrane (Fig. 13). (Smith and Wyckoff, 1950).

The actual time of application and strength of sodium carbonate used are extremely critical and the margin between liberation and dissolution of the virus particles is very narrow. It may be a matter of a few seconds only in some species.

B. Granulosis Viruses

The first record of this type of insect virus disease was made by Paillot (1926a), who described what was probably a granulosis in the caterpillars of the large white butterfly, *Pieris brassicae*, and later (Paillot, 1934) a similar disease in the larvae of the cutworm, *Euxoa segetum*. Steinhaus (1947) first characterized the virus by means of the electron microscope from the larvae of the variegated cutworm, *Peridroma margaritosa* (Haw.). A year later Bergold (1948) observed a similar disease in caterpillars of the fir-shoot roller, *Cacoecia murinana* Hb. Smith and Rivers (1956) described six granulosis diseases from *Euplexia lucipara* L., *Agrotis segetum* Schiff., *Melanchra persicareae* L., *Natada nararia*, *Pieris brassicae* L., and *P. rapae*, and several more are recorded in the literature.

In the granuloses there are no large polyhedral crystals, but their place is taken by the "granule" from which the disease takes its name. The granule is also a crystal (Fig. 14) and usually contains one virus rod, which can be liberated by means of weak alkali in a similar manner to the nuclear polyhedra (Fig. 12). The tissues of affected larvae contain immense numbers of granules which, when stained with Giemsa solution, are just within the limit of resolution of the optical microscope. So far, granuloses have only been recorded from the larvae of Lepidoptera.

The following description of the granulosis disease of *Pieris brassicae* is fairly typical of the group. Under laboratory conditions the onset of the disease is very rapid, and young larvae begin to die within 72 hours of infection; older larvae take a little longer. The first indication of infection is loss of appetite; infected larvae immediately stop feeding and remain quiescent. A pallor next develops, which is most marked in the thoracic region, and death rapidly ensues after this. The larva becomes flaccid and hangs down in a characteristic manner, rather in the shape of an inverted "V." The skin is extremely fragile and ruptures at a touch, liberating the contents of the body which have become almost entirely liquefied. Studies of sections of caterpillars at different stages of infection seem to support Paillot's suggestion that the granules occur in the hypodermis and fat body. Furthermore, it seems as if the virus develops in the nucleus rather than in the cytoplasm. In sections through the fat body of a young larva of *P. brassicae*, 96 hours after infection, the nuclei appear to be full of granules and there are indications

FIG. 8. Transverse section through the mid-gut of a larva of *Lithophane lapidea* infected with a cytoplasmic polyhedrosis. Note the polyhedra filling the lumen of the gut. (Magnification: × 650.)

FIG. 9. Larvae of *Phlogophora meticulosa* killed by a cytoplasmic polyhedrosis. Note the accumulations of polyhedra showing through the skin. (Magnification: × 1¼.)

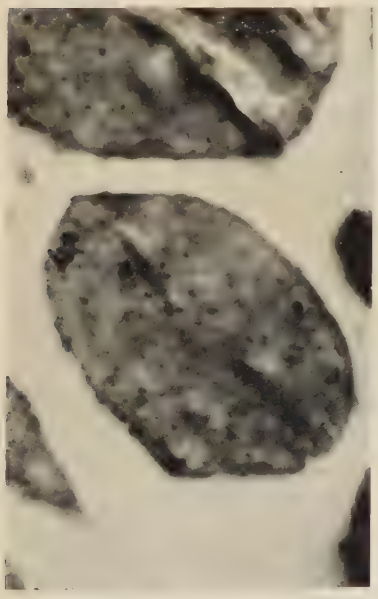


FIG. 10. Section through a cytoplasmic polyhedron from a larva of *S. ligustri*; it is not easy to see the virus particles (compare Fig. 19). (Magnification: × 27,000.)

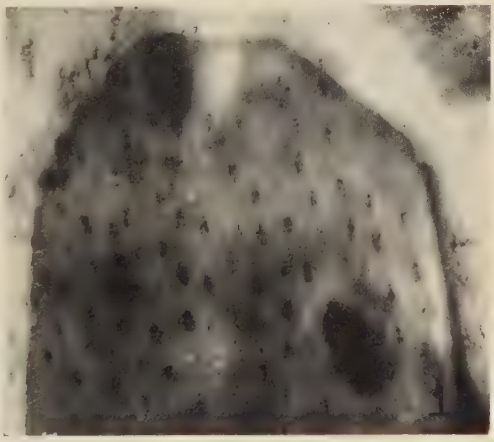


FIG. 11. Part of a section through a cytoplasmic polyhedron from a larva of *Arctia caja*. Note the apparently composite virus particles. (Magnification: × 57,000.)

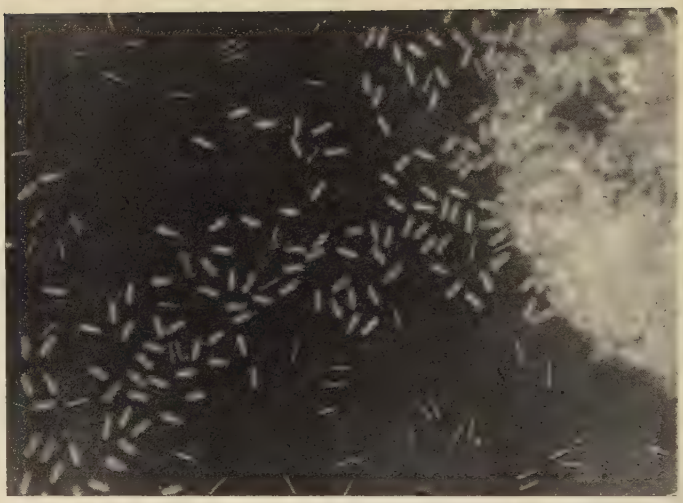


Fig. 12. A nuclear polyhedron from the silkworm, *Bombyx mori*, after treatment with weak sodium carbonate. Note that the crystal has dissolved and liberated many virus rods, some with and some without their capsules. (Magnification: × 17,000.)

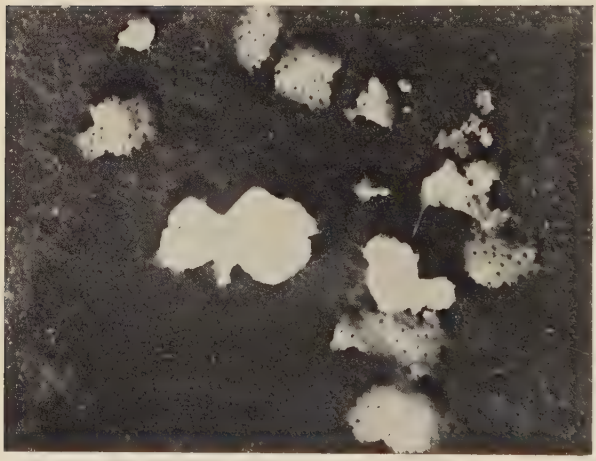


Fig. 13. Cytoplasmic polyhedra from *P. meticulosa* after treatment with weak sodium carbonate. Note sponge like residue and absence of virus particles (compare Figs. 11 and 12). (Magnification: × 14,000.)

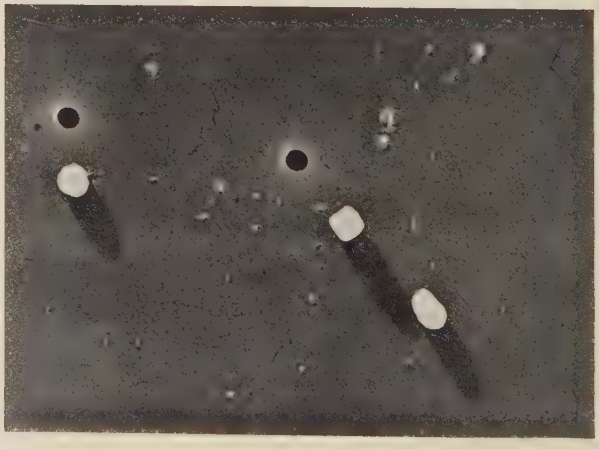


Fig. 14. Three granulosis particles from a larva of *Pieris brassicae*, frozen-dried. Note the crystalline shape (compare Fig. 22). (Magnification: × 15,000.)

(Fig. 14 electron micrograph by R. C. Williams.)

that they rupture, liberating a mass of granules into the cytoplasm of the cell (Fig. 15) (Smith and Rivers, 1956).

C. Viruses without Intracellular Inclusions

As mentioned earlier, the number of viruses so far discovered, occurring freely without crystalline inclusions in the tissues of insects, is not large. There are two in which the virus itself has been well-characterized on the electron microscope, one attacking a lepidopterous larva, *Cirphis unipuncta* Haworth, and the other infecting the dipterous larva, *Tipula paludosa*.

Larvae of *C. unipuncta* infected in late third instar appear swollen and somewhat darker than normal insects. The cuticle of the diseased larvae has a waxy appearance, and in some cases the mid-portion is slightly enlarged. The liquefaction and disintegration characteristic of the nuclear polyhedrosis of the same insect are absent. Deaths from the disease may occur in either the larval or pupal stage. As the disease progresses, the *Cirphis* larvae become sluggish, are soon eating little, and gradually succumb, usually within 6 to 14 days following infection (Wasser, 1952). There seems to be no information as to the site of multiplication of this virus in the insect's body or whether it is nuclear or cytoplasmic in origin. By the analogy of shape, however, this virus, which is very small and apparently spherical, should multiply in the cell cytoplasm.

The second virus of this type, which attacks the larva of the crane fly, *T. paludosa*, is most unusual in many ways and is of great scientific interest. It was first discovered by the Virus Research Unit at Cambridge in 1954 and was later briefly described (Xeros, 1954; Smith, 1954).

The change induced in infected larvae is very striking and enables the disease to be diagnosed with ease. The normal color of these larvae, which are known in England as "leatherjackets," is a brownish gray, while the diseased specimens exhibit a somewhat opalescent blue-indigo. This color is rendered more striking if the insect is placed in a test tube with moistened sides and viewed through the glass. The site of multiplication of the virus is in the cytoplasm of the fat body cells and it is here that the blue color originates. When sections of the diseased fat body are viewed in the electron microscope, it is seen that the cells are filled with the darkly staining virus particles. No virus occurs in the cell nuclei and there are no polyhedra such as would be present in a cytoplasmic polyhedrosis. The amount of virus in the fat body is very great and the blue or violet color is due to the optical effect given by the virus, which actually begins to crystallize in the living insect. The quantity of virus produced by each larva is extremely high and measurements suggest that one-quarter of the dry body weight is converted into virus particles. Pellets formed from this virus, which is known as the tipula iridescent virus (TIV),

are found to have fascinating optical properties. When observed by transmitted light they are generally orange in color, but when examined by reflected light the pellets have a beautiful iridescence with the more noticeable colors in the blue and green regions of the spectrum. The origin of the colors is shown by making thin sections of pellets which have been dehydrated and embedded in methacrylate. On the electron microscope the sections show a somewhat bizarre pattern of particle array (Fig. 16). This suggests that the pellet is made up of a mass of small crystals, each crystal being about 10 μ in diameter.

Purified preparations of tipula iridescent virus tend to crystallize out after standing in the cold. A suspension of the virus in a glass tube, if left undisturbed, will show a layer of small, brilliantly reflecting crystals at the bottom, with a consequent diminution of virus concentration in the supernatant fluid. It has been found possible to photograph in color an array of crystals on the flat surface of a microscope slide. The crystals, photographed at different angles, give an exquisite kaleidoscope effect. (Smith and Williams, 1958).

III. Morphology of Insect Viruses and their Associated Membranes

A. Nuclear Polyhedral Viruses

Insect viruses differ greatly in size and shape; these variations almost equal those occurring in the plant viruses. There are, in addition, to complicate matters, the various membranes and envelopes which in many cases enclose the virus particles.

As we have mentioned previously, the shape of the virus particles from the nuclear polyhedroses seems to be exclusively rodlike, but there is very little homogeneity in the sizes of the virus rods and the size may vary even within one nucleus. This is particularly the case with *Lymantria monacha*, which seems to show the greatest variation in the length of the rods. One individual polyhedral body may contain an almost complete range of virus rods far longer than the average of 290 mμ to less than one-half this length.

Bergold (1953) gives the average dimensions in millimicrons of a number of nuclear polyhedral viruses, of which the following are typical: *Bombyx mori* L. 279 $\times$ 40; *Porthetria dispar* L. 364 $\times$ 41; and *Lymantria monacha* L. 350 $\times$ 57.

Half-length rods, single and in bundles, occur commonly in several species, notably *Lymantria monacha*, *L. dispar*, *Bombyx mori*, and others. These are the so-called spherical developmental forms of Bergold (1953). The proportion of half-length to normal-sized rods varies from one species to another, and appears highest in *L. monacha* and *L. dispar*, with very few half-sized rods in

B. mori and *Abraxas grossulariata*. In sections of *B. mori* infected with its nuclear polyhedrosis, extremely long virus rods are sometimes observed of a size never seen among rods obtained from polyhedra. Similar long rods, which appeared to be breaking up into two more normal-sized rods in the nuclear ring zone, have also been observed.

Bergold (1950) and Bird (1952) have described V-shaped forms which, in the writer's opinion, consist of either two half-length rods within one capsule or two bundles of half-length rods, each bundle within its own inner capsule and the two bundles lying alongside each other at an angle (Smith and Xeros, 1954c).

As regards the outer membranes and capsules of the nuclear polyhedral viruses, there is, first, the intimate membrane, which actually holds the constituent protein and nucleic acid components of the virus. Then comes the inner capsule, which is acquired by the virus rod after its formation and after it has been liberated into the nuclear ring zone, (Smith and Xeros 1954a). Finally, there is the polyhedral crystal with its own encircling membrane.

In the rather atypical nuclear polyhedrosis of *Tipula paludosa*, the inner capsule behaves in a manner quite different from other nuclear polyhedroses. During the formation of the polyhedral crystal, the virus rod without its inner capsule can be seen in the center of a large vesicle (Fig. 17). This vesicle may be the greatly enlarged capsule, as suggested by Xeros (1957), connected with the peculiar elasticity of the polyhedral crystals. On the other hand, sections of the virus particles in the insect's tissues (Fig. 18) sometimes reveal a somewhat similar dilation of the capsule in the absence of polyhedral bodies.

B. Cytoplasmic Polyhedral Viruses

Although there is some variation in the length of virus rods in the nuclear polyhedroses, this does not approach the degree of variation in morphology which occurs in the cytoplasmic polyhedral viruses. The virus particles from three separate cytoplasmic diseases are briefly described to illustrate this diversity of form. The difficulty of isolating some of the cytoplasmic viruses from their enclosing polyhedra has been previously commented upon (Smith and Xeros, 1954c), so it is less easy to make a comparative survey of the virus morphology of this group than with the nuclear polyhedral viruses.

The first example is a cytoplasmic polyhedrosis of the larva of *Sphinx ligustri*, the privet hawk moth. At first, owing to the difficulty of isolating this virus, thin sections were cut of the polyhedra and observed on the electron microscope. As can be seen from Fig. 10, it is difficult to observe virus particles in the sections of the polyhedra. However, after some experimenting with the polyhedra both in bulk and on the electron microscope grid, using very weak alkali for very short periods, the virus was eventually isolated. It

proved to be present in considerable quantities and of an unexpectedly small size, measuring only 12–15 mμ; it is apparently spherical. There is little doubt that this virus can be crystallized; in Fig. 19 a microcrystal can be seen.

The second type of virus comes from a cytoplasmic polyhedrosis of one of the tropical silkmoths, *Antheraea mylitta* Drury. These particles seem to be definitely polyhedral in shape and appear to be 6-sided (see the tipula iridescent virus, p. 383). They measure about 30 mμ in diameter and in Fig. 20 they can be seen arranged in a regular manner within the matrix of the polyhedral body. Figure 21 shows two virus particles at a very high magnification in which the polyhedral shape can be made out.

The third type of virus was first observed in a cytoplasmic polyhedrosis of the larva of *Phlogophora meticulosa*, the large angleshades moth. The virus particles were apparently spherical and measured about 60 mμ in diameter. The peculiarity about this virus however, is that the particles appear to be composite, consisting of a number, usually four, of very small units, each about 15 mμ in diameter. The composite bodies were also found loose in the cytoplasm together with some of the small single units (Smith and Xeros, 1954c). A similar state of affairs exists in the cytoplasmic polyhedrosis of *Arctia caja*, the garden tiger moth. Here again the particles seem to be made up of five or six subunits (Fig. 11) (Smith, 1956).

C. Granulosis Viruses

Quite a large number of granuloses have now been recorded, but so far only from lepidopterous larvae. In every case the virus particle is rod-shaped and closely resembles the virus rods of the nuclear polyhedroses. The dimensions of the virus rods occurring in a granulosis of *Pieris rapae*, which is a typical case, are given as 41–50 × 291–300 mμ. (Tanada, 1953).

The mode of concealment of the virus particle within granule and capsules is rather complicated. As a rule, a single virus rod is thus enclosed, though it is possible there may be two in some cases (Tokuyasu, 1953). The number and arrangement of the enclosing membranes has been worked out in some detail for a granulosis disease of *Natada nararia*, the "nettle-grub" of tea (Smith and Xeros, 1954c). This process was divided into four steps and viewed on the electron microscope. First come the "granules," which are actually minute crystals (Fig. 22a) and opaque to the electron beam. Second, after treatment with weak alkali, the granule collapses on the grid, revealing a rod-shaped body within (Fig. 22b); this is the inner capsule. Third, the virus rod can sometimes be caused to emerge from this capsule (Fig. 22c); and fourth, further treatment with weak alkali dissolves the actual virus content of the rod, leaving behind the intimate membrane (Fig. 22d). This arrangement of occluding membranes can also be demonstrated by thin sectioning

for electron microscopy, which also reveals the crystal lattice of the granule, see Fig. 23.

D. Viruses without Intracellular Inclusions

According to Wasser (1952), the size of the small virus isolated from *Cirphis unipuncta* (Haworth), the cosmopolitan army worm, is approximately 25 mμ in diameter. It is described as a more or less regular, spherical to slightly ovoid body.

The other virus in this category is the tipula iridescent virus (TIV) and its size and shape have been very carefully studied. In air-dried and frozen-dried preparations the virus appears as a five-or six-sided particle and in sections of the particle itself some degree of nonuniform structure can be seen. There appears to be an outer envelope, inside of which there is a relatively transparent region; the central area of the particle is filled with opaque material, tentatively identified with its nucleic acid portion. An unexpected observation is the fairly frequent appearance of six-sided contours when the virus particles are seen in section.

Purified preparations of TIV appear quite monodisperse in the electron microscope, with each virus particle having a diameter of about 130 mμ. Even in specimens dried out of a water suspension in the usual way, and hence suffering the distortions brought about by surface tension, the characteristic contour of the particles is six-sided, rather than circular (Fig. 24). This appearance is quite unique among the known viruses of comparable size; vaccinia virus for example, is brick-shaped and nonuniform in size, while influenza virus appears quite circular and heterodisperse. Since the particles of TIV appear to have six sides, it is reasonable to suppose they are polyhedral in external shape. Other cases of polyhedral-shaped virus particles are known (Williams, 1953) among the bacterial and plant viruses. But up to now it has not been possible to arrive at a convincing notion of the exact form of the polyhedron. An indirect approach is to infer the three-dimensional shape of the polyhedra from the shapes of the shadows found by application of the shadow-casting technique (Williams and Wyckoff, 1954). The large size and regular shape of the TIV particles are suitable for the determination of their full polyhedral shape by analysis of shadow shapes (Figs. 25 and 26). This has been done by Williams, who has shown that the virus particles, when frozen-dried and shadowed with azimuth angles 60° apart, cast two shadows, one five-sided and blunt on its end, the other four-sided and pointed. The only polyhedron which will do this is a twenty-sided figure, so that we can conclude that the three-dimensional morphology of one virus is now known (Fig. 25) and that its shape is that of an icosahedron (Smith and Williams, 1958).

E. The Ultimate Infective Unit

The criticism has been made of a too facile assumption that the various types of particles associated with the different insect virus diseases are the actual virus particles. In the writer's opinion this criticism is not valid, in view of the extreme similarity between these particles and other viruses of proved infectious nature. So far as the nuclear polyhedroses are concerned the criticism does not apply, since Bergold (1953) has demonstrated the high degree of infectivity possessed by the purified rods extracted from the polyhedra. It is true that the parallel experiment with the purified particles from the cytoplasmic polyhedra does not seem to have yet been done. It is known, however, that the polyhedra are extremely infectious; a glance at Figs. 19 and 20 will show how exactly similar the extracted particles are to other viruses. It is perhaps more legitimate to inquire what the ultimate infective unit is and how far the numerous enclosing capsules and membranes influence the infectivity of some viruses.

In certain viruses from cytoplasmic polyhedroses the particle appears to be composite and at very high magnification, a number of apparent subunits are visible (Fig. 11). Experimental evidence as to whether these subunits are infectious has not yet been obtained but they are interesting as affording a possible insight into the build-up of some spherical infectious particles.

IV. Pathological Changes in the Infected Cell and the Development of the Virus Particles

In the nuclear polyhedroses the most striking pathological change is the development of the central chromatic mass or net, referred to by Xeros (1956) as a "virogenic stroma." This central mass has been observed also by earlier workers, but the first electron micrograph of a section through this nuclear net was shown in a polyhedrosis of the larva of the privet hawk moth, *Sphinx ligustri* (Smith *et al.*, 1953). The chromatic mass or net was once thought to be a stage in the further development of a fused aggregation of nucleoli (Mazzocchi, 1908; Glaser, 1927). Another view was that the net was formed primarily by the fusion of chromatin granules of the pathological nucleus (Paillot, 1926c; Heidrenreich, 1940). However, according to Xeros (1955), who made a detailed histological study of a number of nuclear polyhedroses, no evidence was found that the chromatic mass had been formed from the chromatin. He concludes that the chromatic masses or nets do not arise by fusion of chromatin granules but are produced *de novo*. It seems clear that this chromatic mass is the site of the development of the virus rods, but about the exact method of development of the virus rods there is still a good deal of uncertainty. Bergold (1950, 1953) postulated a life cycle

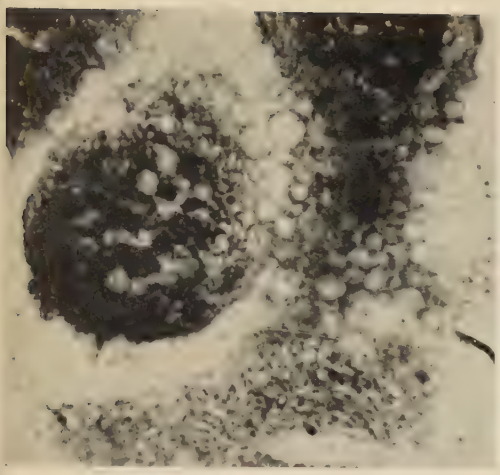


FIG. 15. Section through the fat body of a larva of *Melanchra persicariae* infected with a granulosis virus. Note the nucleus on the right apparently liberating granules into the cytoplasm. (Magnification: × 660.)

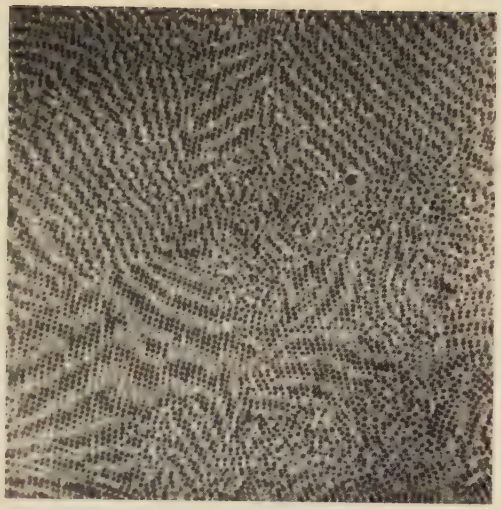


FIG. 16. Section through a methacrylate-embedded pellet of the tipula iridescent virus. The pattern results from a transection made through small crystalline regions oriented at random. (Magnification: × 5800.)

(Fig. 16 after Williams and Smith, 1957).

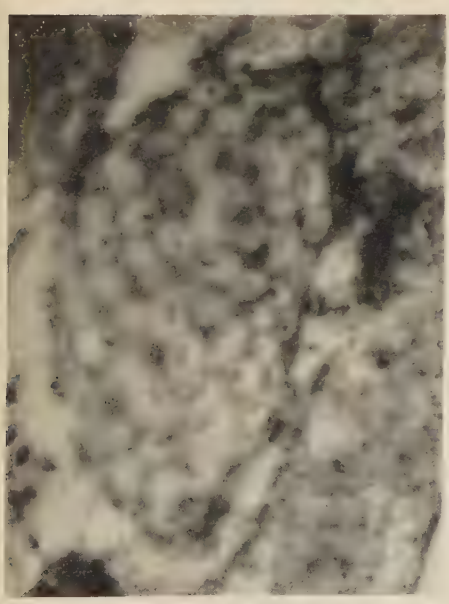


FIG. 17. Section through part of a nucleus of a blood cell from the larva of *T. paludosa* infected with its polyhedrosis. Note the peculiar vesicle surrounding each virus rod. (Magnification: × 20,000.)

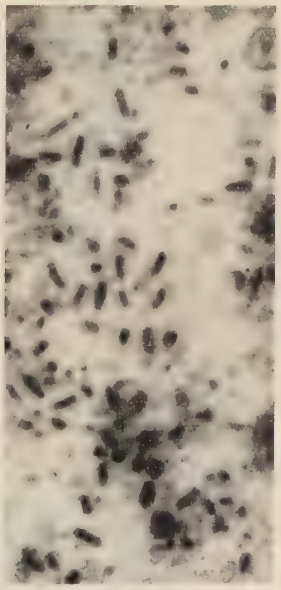


FIG. 18. Sections through virus particles from the same disease as in Fig. 17. Note a similar dilatation of the membranes surrounding the particles. (Magnification × 40,000.)

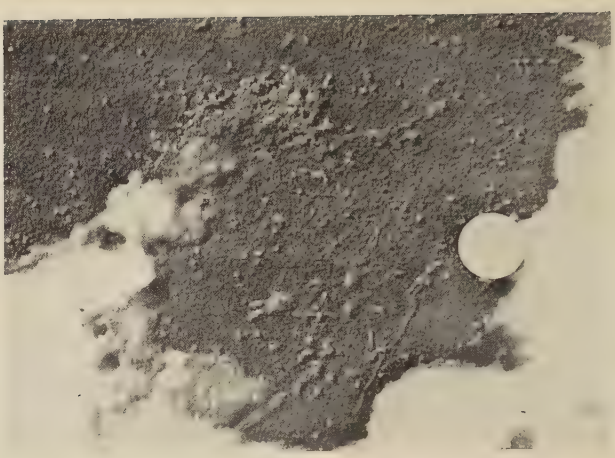


Fig. 19. Very small virus from a cytoplasmic polyhedrosis of *S. ligustri*. Note the formation of a micro-crystal. (Magnification: × 40,000.)

Fig. 20. Virus from a cytoplasmic polyhedrosis of *Antheraea mylitta*. Note the regular packing of the virus particles and their polyhedral outline. (Magnification: × 30,000.)

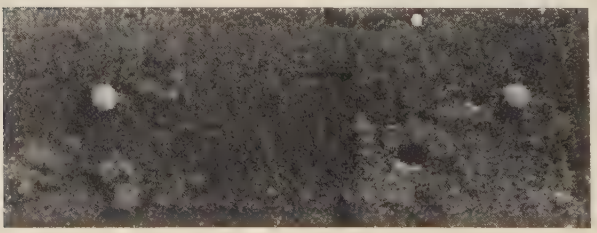
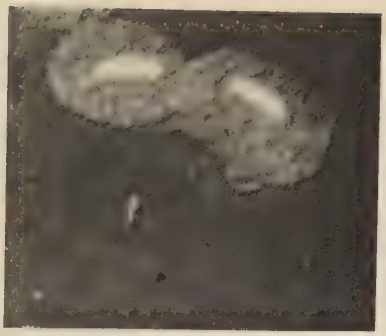


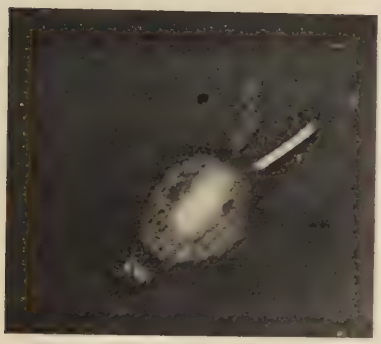
Fig. 21. The same virus as in Fig. 20 at high magnification to show the six-sided contour. (Magnification: × 60,000.)

(a)

(b)



(c)



(d)

FIG. 22. Four stages in the breakdown of a granule from a larva of *Natada nararia*: (a) the untouched granules; (b) after treatment with weak sodium carbonate, showing the collapse of the outer granule and the inner capsule; (c) a virus rod outside the capsule; (d) dissolution of the virus rod showing the intimate membrane. (Magnification: × 30,000.)

FIG. 23. Thin section of granules from a larva of *P. brassicae*, with one virus rod cut longitudinally, showing the inner capsule, and one virus rod cut transversely. Note also the apparent molecular lattice of the outer granule. (Magnification × 80,000.)

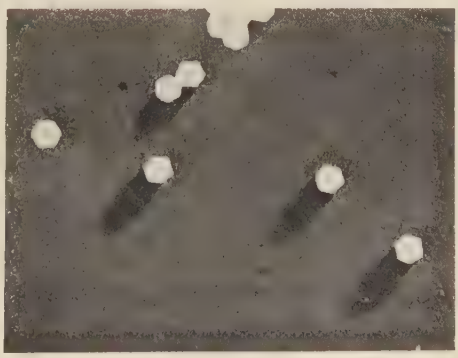


FIG. 24. Frozen-dried particles of the tipula iridescent virus showing the six-sided shape. (Magnification: × 40,000.)

(Figs. 24, 25, 26 after Smith and Williams 1958.)

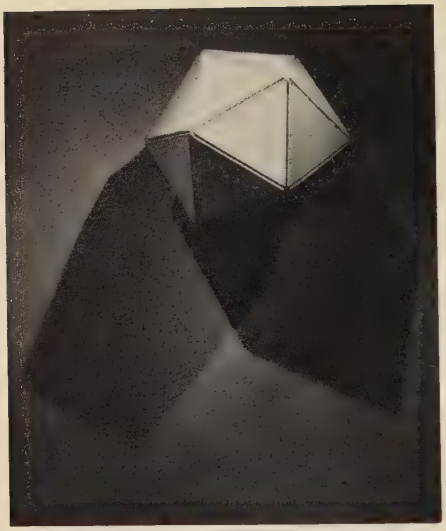


FIG. 25. A model of an icosahedron doubly shadowed. Note two shadow shapes which can be cast simultaneously only by an icosahedron in this orientation.

FIG. 26. Frozen-dried particles of the tipula iridescent virus doubly shadowed. Note similarity to shadows in Fig. 25. (Magnification × 50,000.)

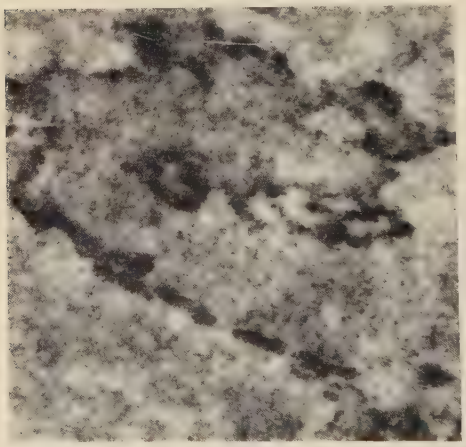


FIG. 27. Section through the nucleus of a blood cell from *T. paludosa* infected with its polyhedrosis. Note the apparent development of the virus rods in the central chromatic mass. (Magnification: × 28,000.)

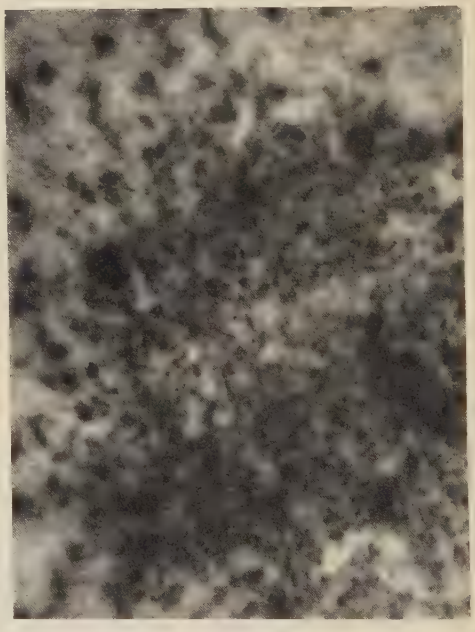


FIG. 28. Later stages of the same disease as in Fig. 27. Note the fully formed virus rods, some with enclosing membranes. (Magnification: × 25,000.)

for insect viruses based on a number of apparent "developmental forms" collected at random from dissolved polyhedra observed on the electron microscope. He considered that the virus rod appeared first as a minute spherical body, which gradually increased in size to form an elongated, curved body surrounded by a membrane. Later the rod straightened out, ruptured the membrane, and escaped, leaving an empty spherical membrane behind.

Bird (1957) supports this theory of a life cycle; he has investigated a nuclear polyhedrosis of the sawfly, *Neodiprion pratti banksianae* Roh., and considers that a study of thin sections of infected nuclei suggests a cycle of virus development, commencing with the attachment of rod-shaped particles to strands of chromatin. According to Bird, the chromatin is then converted to virus in the form of minute spherical bodies surrounded by membranes that increase in size to form rods. The rods may escape from their developmental membranes to repeat the cycle, or rods and spheres may be occluded by protein material to form polyhedra, in which case virus development ceases.

On the other hand, Xeros (1956) considers that the nuclear nets or virogenic stromata become increasingly proteinaceous and Feulgen-positive as they grow and develop. Morphologically they are net works, and virus rods differentiate within vesicles in their cords. These begin as fine rodlets about 60 Å × 1200 Å in size and increase *in situ* to their final size of 280 Å × 2800 Å. They are then set free from their vesicles into the pores of the net by disruption of the surrounding cord material and may ultimately reach the ring zone between the centrally placed virogenic mass and the nuclear membrane. The freed virus rods become enveloped by independently formed and still growing capsule membranes, within which capsule protein is deposited. The encapsulated rods then become occluded within crystalline protein polyhedra, which arise and grow in the ring zone and later in the enlarged pores of the infected nucleus.

In thin sections, cut by the writer, of the nuclei of blood cells of *Tipula paludosa*, infected with its characteristic polyhedrosis, what appear to be immature virus rods can be seen in large numbers round the periphery of the chromatic mass (Fig. 27). At a later stage of the disease, sections show the fully formed rods, in some cases with their capsule membranes, differentiated out of the central mass (Fig. 28).

If we accept Bergold's thesis that insect viruses are organisms with a "life cycle," we are faced with the anomaly that this life cycle applies only to one group of insect viruses, those of the nuclear polyhedroses. There is still the very large group of cytoplasmic viruses, some of which are of extremely small size and are comparable to the smallest plant viruses. As we have seen, certain of these insect viruses are crystallizable and the particles themselves

are minute polyhedral crystals. It would be extremely difficult to postulate any form of developmental cycle for particles of this nature.

Xeros (1956) considers that the virus particles of cytoplasmic polyhedroses arise in "virogenic stromata" essentially similar to those of the nuclear polyhedroses. He has studied a cytoplasmic polyhedrosis in a processionary caterpillar or "army worm," *Thaumatopoea pityocampa*, in which he describes virogenic stromata with a micro-net structure. In the cords of the stromata virus bodies arise which are spherical, with an extremely dense center about 35 mμ in diameter and a less electron-dense cortex about 80 mμ in diameter. When the virus bodies have been formed, the cord material around them disrupts and liberates them into the larger pores formed as a result of dissolution of the cords. The freed virus bodies become occluded in the polyhedra.

V. Latent Infection in Insects and its Bearing on the Cross-Transmission of Viruses

There is a good deal of confusion caused in the study of latent infections by a lack of uniformity in the use of such words as "latent," "inapparent," "subclinical," and "masked." A symposium on the subject was recently held at Madison, Wisconsin, and the chief findings were reported by Andrewes (1957). It was suggested that the words "latent virus" should not be used but that "inapparent infection" at the host-parasite level would cover the whole field of infections which give no overt signs of their presence. "Latent infection" denotes those cases of inapparent infection which are chronic and in which a certain host-virus equilibrium is established. At the cell-virus level, Dulbecco's term "moderate virus" was approved to denote a virus which grew in a cell while still permitting its continued survival and multiplication; "cytocidal" described one which killed it; "submoderate" covered intermediate cases.

There is no doubt that large populations of insects, particularly lepidopterous larvae, carry latent infections; this fact is liable to cause much confusion in experiments in the cross-transmission of insect viruses.

For many years the opinion was held that insect viruses were extremely specific and that true examples of transmission between different species were unknown; examples of apparent cross-infection in the literature were regarded as unproved. In 1953 a paper was published (Smith and Xeros, 1953b) in which a large number of experiments, involving apparently genuine cross-transmission of viruses, was described. The alternative possibility of stimulation of latent infection in some cases was, however, admitted. Up to that date the term "insect virus" referred mainly to the nuclear polyhedral viruses and possibly to those of the granuloses. With the discovery of the distinct group of cytoplasmic polyhedroses the situation became more complicated.

The following experiments on cross-transmission with the larvae of *Sphinx ligustri*, the privet hawk moth (Smith and Xeros, 1953a), give a fairly characteristic picture of what happens with this type of experiment.

Six similar batches of third and early fourth instar larvae of *ligustri*, which appeared perfectly healthy, and among which no deaths from polyhedrosis had occurred, were fed on the same day with nuclear polyhedra from the following lepidopterous larvae, chosen entirely at random: *Telea polyphemus*, *Lymantria dispar*, *Philosamia ricini*, *Panaxia dominula*, and *Cycnia mendica*. The *ligustri* larvae infected with virus from *mendica* and *dominula* died on the seventh and eighth days after infection with a typical nuclear polyhedrosis. The polyhedra were nonstaining and square in shape. The larvae infected with the *polyphemus* virus died on the eighth day with identical symptoms, but the polyhedra in this case were either many-sided or triangular in shape and not square. The deaths were extremely regular, occurring together over two or three days, and the symptoms were classic. One larva only, one of those dying on the ninth day, showed some staining cytoplasmic polyhedra among the nuclear type. Of the twelve *ligustri* larvae infected with virus from *ricini*, two died on the tenth day, four on the eleventh, one each on the twelfth and thirteenth days, one on the eighteenth, and one each on the twenty-ninth and thirty-second day after infection. The first six larvae to die had fairly typical symptoms of a nuclear polyhedrosis. Their polyhedra were nonstaining, with a tendency to a square shape. The other six larvae commenced to die on the eleventh day and subsequently; two of these had cytoplasmic polyhedroses only, while the other four had both types of infection.

Those *ligustri* larvae infected from *dispar* all died of a cytoplasmic polyhedrosis, and none developed a nuclear disease. The control larvae for this experiment also died similarly.

The conclusion we draw from this type of result is that there was genuine cross-transmission of a foreign nuclear polyhedral virus; but, in addition, there was a latent cytoplasmic polyhedrosis. This latter point has been amply confirmed by subsequent observations on *S. ligustri*. Provided the incubation period of the nuclear virus was short (*mendica*, 7 days, *dominula* and *polyphemus*, 8 days) the foreign virus was able to establish itself early and kill the larva before any appreciable quantity of gut polyhedra developed at the molt to the fifth instar. In the case of the *ricini* infections, the incubation period of 11 days was slightly longer and there appeared to be a suppression of any great development of nuclear polyhedra in those older larvae in which the cytoplasmic disease had got a good start before the nuclear one could do so. With the *dispar* virus one can conclude either that it is not cross-transmissible to *ligustri* or else that the cytoplasmic disease suppressed its getting a foothold. In fact, we can now go further and say that where a latent cytoplasmic infection is present this is almost invariably stimulated to development

by the introduction of a foreign nuclear virus. The reverse phenomena, stimulation of a nuclear virus by introduction of a foreign cytoplasmic virus, does not seem to occur. Some experiments with the larvae of the winter moth, *Operophtera brumata*, are of interest in this connection. Failure to find a naturally occurring virus disease of this larva led to attempts to infect it with a nuclear virus from the butterfly larva, *Vanessa cardui*. This induced a high percentage of mortality in the winter moth larvae, all the controls remaining healthy. The disease which resulted, however, was a cytoplasmic one and, once stimulated into activity could be transmitted indefinitely in series (Smith and Rivers, 1956).

It may be that the reason for the frequent stimulation of a cytoplasmic virus by a foreign nuclear virus is connected with the location of the cytoplasmic virus in the cells of the mid-gut. In this position it is likely to come rapidly into contact with the foreign virus swallowed by the larva. There is little doubt that the number of latent infections with cytoplasmic viruses is extremely high and, in the writer's opinion, higher than the number of latent nuclear polyhedroses.

There are other methods by which latent polyhedral viruses can be stimulated to action, notably by feeding with certain chemicals and by keeping the larvae under unsuitable conditions.

A few other examples of apparently genuine cross-transmission may be quoted. A nuclear polyhedral virus from *Vanessa cardui* is easily transmissible to *Aglais urticae* and *V. io* and is intertransmissible between all three species.

With the granulosis virus of the large white butterfly, *Pieris brassicae*, transmission is easily achieved between this species, *P. rapi*, and *P. napi*. It is difficult to say, at this moment, whether it is always the same granulosis virus which is involved, since there is no means of differentiating between them until they can be tested serologically. Steinhaus (1952) has demonstrated a similar cross-transmission of a granulosis virus between species of *Colias*.

It is possible that in the case of the three species of white butterfly, *Pieris*, the granulosis virus may change slightly after passage of one or other of the species. It has been found (Smith and Rivers, unpublished) that a considerable resistance on the part of *P. brassicae* can develop to infection with the granulosis virus, but after passage of the virus through *P. napi*, this resistance breaks down.

Attempts to stimulate development of the granulosis, which is frequently latent in *Pieris* spp., by feeding with foreign polyhedral viruses have given negative results.

VI. Methods of Transmission of Insect Viruses

There can be little doubt that the two main methods of transmission of insect viruses are by ingestion of contaminated food and by the inheritance of

infection through the parent. This statement should perhaps be modified slightly in so far as it is known to apply to the polyhedroses and granuloses; there is not yet sufficient evidence to say definitely that the viruses without intracellular inclusions are similarly transmitted. We know that TIV is spread by ingestion orally, in the laboratory and presumably also in the field; there is a slight amount of evidence that there may be latent infection with the same virus.

Transmission of the polyhedral viruses and those of the granuloses through a given population of insect larvae during the current season is undoubtedly by the ingestion of contaminated foliage. So far as the nuclear polyhedroses are concerned the disease itself greatly facilitates the spread of the polyhedra. The skin, being one of the organs attacked, quickly becomes extremely fragile and easily ruptures, scattering the polyhedra in the liquefied body contents over the foliage. This process is helped by the wind and the rain which carry the polyhedra still further afield; the fact that the polyhedra are quite resistant to the weather is very important in the spread and overwintering of the virus. The same principles probably hold in the spread of the granuloses, since the skin is also attacked and the liquefied body contents are similarly spread abroad. There is some evidence, however, that the longevity of the granules is much less than that of the polyhedra; the granules from *Pieris* spp., for example, seem to lose much of their infectivity after storage for one winter.

An interesting fact in regard to the granulosis disease of the large white butterfly, *Pieris brassicae*, is the apparent attraction of the liquefied cadavers for the healthy caterpillars, which can often be seen feeding greedily on the bodies with, of course, disastrous results.

The situation regarding the cytoplasmic polyhedroses is slightly different, since the skin of the affected larvae is not attacked and in consequence there is no general liberation of polyhedra. In this case, the polyhedra are excreted in large numbers with the feces and the food plant in consequence is heavily contaminated. This fact is easily demonstrated by examining smears of the droppings of larvae with cytoplasmic polyhedroses, as in *Operophtera brumata*, the winter moth, for instance, or by examining sections of the gut of infected larvae (see Fig. 8).

Since the polyhedra are capable of retaining viable virus within them for many years, fifteen years in the case of the silkworm, *Bombyx mori*, it is fair to assume that infective material will remain over winter on contaminated food plants. This has been proved on more than one occasion, for example, with the Great Basin tent caterpillar, *Malacosoma fragile* (Stretch). In 1953, a number of trees in an abandoned orchard in California was sprayed with a polyhedral virus from this species and a second block of trees was left untreated. It was observed that a number of larvae died of the disease on the

treated section. In the spring of 1954, from 60 to 80 % of the larval population in the treated block died of polyhedrosis. No evidence of virus was found in the untreated block (Clark, 1956).

The second most important method of insect virus dissemination is by passage of the virus through the egg; this applies to both types of polyhedral viruses and is probably more frequent with the cytoplasmic polyhedroses. Sudden outbreaks of polyhedroses in areas where the disease was unknown, (Tanada and Beardsley, 1957), and attacks under conditions where external contamination was impossible are certainly due to the stimulation of a latent infection. Geneticists and others who breed large numbers of lepidopterous larvae in captivity are only too familiar with this phenomenon.

There has been some disagreement on the exact mode of transovarial transmission as to whether the virus is inside the egg or is carried mechanically attached to the outside of the shell. There are two facts which suggest that the virus is not merely an external contamination of the outside of the egg: one is the sudden appearance of a polyhedrosis in caterpillars which have been reared through a number of generations under conditions where external contamination is ruled out. It is unlikely that virus on the outside of an egg would go through several generations in a latent infection. If the virus can remain latent throughout the larval and adult life, there seems no reason why it should not also be latent in the egg stage. The second fact against the theory of external contamination of the egg surface is the discovery that young larvae may die of a polyhedral disease even before they have left the egg shell (Smith *et al.*, 1953).

There are one or two miscellaneous agents which may possibly help to spread virus infections of insects and these are briefly dealt with. Predacious insects may be instrumental in carrying the polyhedra around, either on their mouthparts or possibly by contamination with their feces. Blowflies and similar insects which are frequently attracted by and feed on the cadavers of insect larvae which have died of virus infections are also potential vectors. The same also probably applies to birds, and, as previously mentioned, the wind and the rain no doubt play a part in distributing the polyhedra. Some recent work by Franz *et al.* (1955) is relevant in this connection. They state that after passing through the intestinal canal of the predatory bug, *Rhinocoris annulatus* L., and of the robin, *Erithacus rubecula* L., the polyhedral viruses of the pine sawfly, *Neodiprion sertifer* (Geoffr.), proved to be still infectious in experiments carried out with the specific host.

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